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REMARKS

Applicant respectfully requests reconsideration of the present application in view of the foregoing amendments and in view of the reasons that follow.

Claims 2, 4-20 and 46 are pending. Claims 4, 7, 9, 11 have been amended to more clearly define the present invention, and should not be construed as the surrender of any subject matter. Applicants reserve the right to file one or more continuing applications on any canceled subject matter. The amended claims have support in the original claims and specification as filed.

This amendment changes claims in this application. A detailed listing of all claims that are, or were, in the application, irrespective of whether the claim(s) remain under examination in the application, is presented, with an appropriate defined status identifier.

Rejections under 35 U.S.C. § 112, first paragraph

Claim 20

Claim 20 is rejected by the Examiner as allegedly only being enabled for the *in vitro* administration of the AAV Rep78 mutant for replication studies and it not enabled for *in vivo* therapeutic uses. The Examiner states that he has considered Dr. Hermonat's declaration and characterizes it as providing evidence of a correlation between infection with wild-type adeno-associated virus and a reduced incidence of cervical cancer in the general population. However, the examiner alleges that it does not evidence that a mutated AAV vector can be administered *in vivo* so that it is therapeutically effective for treating cancer or other papillomavirus (PV) associated diseases. The Examiner considers that the therapeutic efficacy of viral vectors administered *in vivo* is unpredictable, and that the specification needs to provide detailed teachings of how to make and administer the claimed viral vectors to treat PV disease or cancer.

Applicant respectfully disagrees with the Examiner's position and maintains that claim 20 is enabled by the specification so that a person skilled in the art could practice the present invention without undue experimentation. Applicants submit that the present

specification provides sufficient disclosure to support claim 20 in combination with what is known in the prior art. Firstly, there have been AAV gene therapy trials that show that AAV can be administered *in vivo*. For example, several press releases from Avigen in 1999 (Attachment 1) disclose the success of delivering the gene for Factor IX using an AAV based on work preformed at Children's Hospital of Philadelphia and Stanford University Medical Center that showed indications of therapeutic benefit. This same study is reported by an abstract in March 2000, by these research groups reporting success in using gene therapy to treat hemophilia B in 3 patients by delivering the gene encoding Factor IX with AAV. Additionally, other Avigen press releases disclose successfully delivering the gene encoding Factor IX using AAV to dogs (Attachment 2). Additionally, Avigen scientists showed that a single administration of AAV carrying the gene for Factor VIII produced physiological levels of biologically active Factor VIII in the plasma of animals and showed that significant levels persisted in these animals for nearly two years (Attachment 3). Another group from Avigen and Berkeley National Laboratory presented research results that dopamine activity could be restored in a primate model of Parkinson's disease following treatment with AAV carrying the gene encoding dopamine (Attachment 4). As can be seen by these disclosures of successful use of AAV to introduce genes into animals and humans, applicant submits that therapeutic efficacy of AAV is predictable to the skilled person. Applicants also refer the Examiner to U.S. patents, such as 5,670,488, disclosing AAV for gene therapy uses, such as cystic fibrosis.

The claimed AAVRep78 mutant which is administered in claim 20 binds to at least one DNA sequence obtained from a papillomavirus and the mutant's DNA binding is enhanced as compared to the binding of the corresponding wild-type AAVRep78 protein as set forth in SEQ ID NO:6 to the DNA sequence. Thus, this claim focuses on the AAVRep78 mutants that bind to papillomavirus DNA better than the AAVRep78 wild-type. These mutants are considered to be a Rep78 mutant only by the substitution of one or more amino acids for the amino acids in the wild-type Rep78. Furthermore, these mutants possess the major attributes of the full length AAV genome (FLAG) Rep78 by virtue of their ability to replicate as the wild-type AAV that contains a wild-type Rep78 gene. See for example, Figure 11 as described on pages 26, lines 10-18 of the specification, which shows the

replication of AAV Rep78 192^{HG}. In Figure 14, it is shown that the AAVRep78 192^{HG} mutant with the one amino acid change is able to more strongly inhibit HPV-induced oncogenic transformation and HPV-16 p97 promoter activity than wild-type AAV (containing a wild-type Rep78). Therefore, AAVRep78 192^{HG} has an altered biochemistry as compared to wild type AAVRep78 by being able to bind more strongly to DNA (see Figure 12) and inhibit promoter activity (AAV p5) more strongly (see Figures 10). Thus, Applicant submits that the AAVRep78 mutant is a replicating AAV as is the wild-type AAV but with enhanced inhibitory activity against HPV. This is so, particularly in view of the data presented in Figure 14 of the present invention, which uses the focus formation test, which is widely accepted as a measurement of and a model for oncogenic potential *in vivo*.

Applicants submit that treatment with AAV is predictable as supported by the known use of AAV as a vector in gene therapy as supported by the attachments discussed above. Additionally, the previously submitted declaration by Dr. Hermonat and the supporting Coker *et al.* publication showed that AAV containing the wild-type Rep78 protein inhibits papillomaviruses. As noted in the previous response, the Coker *et al.* publication disclosed in the "INTRODUCTION" that AAV is able to inhibit papillomavirus oncogene expression, papillomavirus-mediated transformation and papillomavirus replication. As commented on in the last sentence on page 83, second column of Coker *et al.*, these inhibitory effects have been mapped to the AAV Rep78 protein. Further, other publications in support of the usefulness of AAV Rep78 were provided previously. Dr. Hermonat's declaration also provides additional publications that are cited in Coker *et al.*

Additionally, Dr. Hermonat comments that the *in vitro* data presented in the present application supports the disclosed novel AAVRep78 mutants, such as the AAVRep-192^{HG}, which bind more strongly to specific DNAs as compared to the wild-type AAVRep78 protein. These stronger DNA binders are identified as useful for treating cancer. See the specification, page 26, lines 19-22. Additionally, applicants provide a publication by Storey *et al.* (Attachment 5) that shows in Table 1 that HPV-16 and HPV-18 DNA cause focus formation using baby rat kidney cells while HPV-6 and HPV-11 DNA do not. These results support a direct correlation with what occurs *in vivo* where HPV-16 and -18 have been identified as associated with cancer and HPV-6 and -11 have not. The focus formation is widely accepted

as a measurement of and a model for oncogenic potential *in vivo*. Dr. Storey comments at the end of the first column on page 1819 that

> "[t]here is a striking parallel between the *in vivo* malignancy of HPV-associated lesions and the activity of the corresponding HPV type in our co-transformation assay."

Additionally, a publication by Tsunokawa *et al.* discloses that HPV-16 DNA induced malignant transformation of NIH 3T3 cells (See attachment 6).

Therefore, in view of all of the information, publications and arguments provided above, applicant submits that claim 20 is enabled, and as a result, it is requested that this rejection be withdrawn.

Claims 2, 4-20 and 46

Claims 2, 4-20 and 46 are rejected because the Examiner alleges that the specification, while being enabling for an AAVRep78 mutant which demonstrates enhanced binding to HPV16 and decreasing binding to itself, is not enabled for increased or decreased binding to HIV and oncogenes as compared with wild type. The Examiner states that the specification does not demonstrate either decreased or enhanced binding to either HIV or to an oncogene. Applicant disagrees with the Examiner's position and directs him to page 5, beginning at line 5, where the many proteins that AAVRep78 regulates are disclosed as follows:

> AAV Rep78 regulates a variety of heterologous genes. C-H-*ras* (Katz, *et al.*, *Cancer Research* 46:3023-3026 (1986); Hermonat, P.L., *Cancer Research* 51:3373-3377 (1991); Khleif, *et al.*, *Virology* 181:738-741 (1991)), c-*fos* (Klein-Bauernschmitt, *et al.*, *J. Virol.* 66:419-4200 (1992); Hermonat, P.L., *Cancer Letters* 81:129-136 (1994)), c-*myc* (Klein-Bauernschmitt, *et al.*, *J. Virol.* 66:419-4200 (1992); Hermonat, P.L., *Cancer Letters* 81:129-136 (1994)), and the HIV long terminal repeat (HIV-LTR) (Rittner, *et al.*, *J. Gen. Virol.* 73:2977-2981 (1992); Antoni, *et al.*, *J. Virol.* 64:396-404 (1991)) are down-regulated by AAV Rep78, while the c-*sis* promoter is up-regulated (Wonderling, *et al.*, *J. Virol.* 70:4783-4786 (1996)). Still other genes are not affected, such as the murine osteosarcoma virus long terminal repeat (MSV-LTR)(Hermonat, P.L., *Cancer Research* 51:3373-3377 (1991)) and

> the human β-actin promoter (Horer, *et al.*, *J. Virol.* 69:5485-5496 (1995)). The largest of 4 products encoded by the AAV *rep* open reading frame (Mendleson, *et al.*, *J. Virol.* 60:823-832 (1986)), AAV Rep78, is required for AAV DNA replication (Hermonat, *et al.*, *J. Virol.* 51:329-333 (1984); Tratschin, *et al.*, *J. Virol.* 51:611-619 (1994)) and for AAV gene regulation (Labow, *et al.*, *J. Virol.* 60:251-258 (1986); Tratschin, *et al.*, *Mol. Cell. Biol.* 5:3251-3260 (1986)). AAV Rep78 carries out a range of biochemical activities which are necessary for its biological phenotypes (Im, *et al.*, *Cell* 61:447-57 (1990); Ni, *et al.*, *J. Virol.* 68:1128-1138 (1994)), including binding to promoter DNA (McCarty, *et al.*, *J. Virol.* 74:4988-4997 (1994); Batchu, *et al.*, *Cancer Letters* 86:23-31 (1994); Wonderling, *et al.*, *J. Virol.* 71:2528-2534 (1996)), and to a variety of cellular proteins (Hermonat, *et al.*, *Biochem. and Molec. Biol. Internat.* 403:409-420 (1997)), including the transcription factors Sp1 (Hermonat, *et al. Cancer Research* 56:5299-5304 (1996); Pereira, *et al.*, *J. Virol.* 71:1747-1756 (1997)), TBP (Hermonat, *et al.*, *Virology* 245:120-127 (1998)), and itself (Weitzman, *et al.*, *J. Virol.* 70:2440-2448 (1996), Hermonat, *et al.*, *FEBS Letters* 401:180-184 (1997), Smith, *et al.*, *J. Virol.* 71:4461-4471 (1997)).

Applicants provide the Batchu and Hermonat publication, (FEBS Letter, Vol. 367) (Attachment 7) cited in the present specification as disclosing the inhibitory effect of AAV Rep78 on the HIVI long terminal repeat sequence (LTR). Additionally, Batchu, *et al.*, *Cancer Letters* 86:23-31 (1994)(Attachment 8), also cited in the specification, discloses that AAVRep78 inhibits the oncogene, *ras*. Additionally, AAVRep78 inhibits other oncogenes, such as *myc*, *fos*, and *jun*. The specification as filed discloses many publications that show the effect of the binding of AAVRep78 to the many genes disclosed in the specification. Further, the present specification provides an assay to determine the extent of binding of AAVRep78 to DNA fragments. The specification also provides the entire sequence of the complete AAV-2 genome and Figure 16 provides the amino acid sequence of the wild-type AAVRep78 amino acid sequence. A person skilled in the art can mutate one or more of any one of the amino acid sequences of this disclosed sequence to create mutants as disclosed beginning on page 19 of the present specification. These AAVRep78 mutants that are prepared can then be tested in the disclosed assay for the binding to specifically identified heterologous genes. Nothing more than trial and error experimentation is required to prepare

and test these AAVRep78 mutants. With applicant's specification in hand, undue experimentation is not required to carry out the present application to its full scope. Therefore, applicant respectfully disagrees with the Examiner because there is sufficient guidance for a person skilled in the art to practice the present invention using the disclosure of the specification. In view of these supported arguments, applicant requests withdrawal of the present rejection of claims 2, 4-20 and 46.

Claim 11

Claim 11 remains rejected as not complying with the written description requirement. Claim 11 is directed to AAV Rep-77^{LG} and AAV Rep-79^{FA}. The Examiner has dismissed applicant's arguments and has interpreted them as somehow supporting the Examiner's position that the reproduction of the identical virus is unpredictable. The Examiner again asks for evidence of public availability of the starting material.

Applicant again respectfully disagrees with the Examiner's request that a deposit is necessary. Applicant has provided the complete nucleic acid for the AAV2 (Figure 15) and the nucleic acid encoding the amino acid sequence of the AAVRep78 wild-type protein (Figures 16). Applicant reiterates all of the arguments presented in the previous response and again states that the DNA sequence of the entire AAV2 is disclosed in the present application. No deposit is necessary. Additionally, the same sequence is disclosed in Srivastava *et al.* which is disclosed in the present application and is present in the National Library of Medicine NCBI Sequence site as evidenced by the attached sequence (Attachment 9). If applicant had not provided the sequence of the AAV2 genome, then the Examiner could request whether the present AAV had been deposited but as the sequence is disclosed in the present application, known and published in a scientific paper and available through on-line sequence searches, applicant submits that there is no need to provide evidence of public availability.

To reiterate the present and previous arguments, all of the AAV Rep78 mutants can be prepared from the wild-type AAV Rep78 protein as disclosed in the present application. Figure 15A-C discloses the nucleotide sequence encoding AAV Rep78 as nucleotides 321-

2186 and Figure 16 provides the corresponding amino acid sequence of the AAV Rep78 protein. Thus, the specification provides the sequences needed to prepare the AAV Rep78 mutants. The specification discloses the preparation of these mutants using known methods. Applicant submits that knowing the AAV Rep78 protein nucleic acid and amino acid sequences, a person skilled in the art can follow the specification using standard methods to modify a known amino acid sequence/nucleotide sequence, to prepare the mutants with the desired modifications from the wild-type AAV Rep78 nucleic acid and amino acid sequences, such as a single amino acid change. See the specification on page 19, lines 15 to page 20, line 13.

Applicant submits that the identical virus can be prepared by using the disclosed DNA sequence to do so. Additionally, the amino acid and nucleic acid sequences of AAV Rep 78, methods of making the AAV Rep mutants and methods for assaying for their binding is disclosed in the present application and it would not require undue experimentation to make other AAV Rep 78 modified proteins using the disclosure in the specification.

As argued above, the amino acid and nucleic acid sequences are known and provided in the specification, figures and Sequence Listing. Plasmids containing a specific AAV Rep 78 mutant can be prepared as disclosed. All of the manipulations to create other mutant proteins from known sequences are well within the skill of the artisan. Thus, a person of skill in the art would be able to prepare the claimed AAV Rep 78 mutant using the guidance in the specification for making AAV Rep 77^{LG}, 79^{FA}, and 192^{HG} modified proteins using the known sequence of AAV2 which includes the AAVRep78 sequence.

Applicant submits that a deposit is not necessary as the nucleic acid and amino acid sequences are disclosed in the specification, in a publication and publicly available online . Applicant requests that the Examiner reconsider his position and withdraw this rejection.

Rejections under 35 U.S.C. § 112, second paragraph

Claims 7, 14, 15, 17and 18

Claim 7 remains rejected as being indefinite because the Examiner alleges that it is unclear how long the truncated version of the protein is. The Examiner is referred to the specification on page 13, beginning on line 14, where "the smallest truncated AAVRep78 mutant" that is still capable of binding to the target DNA to inhibit the papillomavirus or oncogene as compared to the corresponding wild-type AAVRep 78 protein defines the smallest truncated mutant encompassed by the claim. Applicant submits that the claims define a functional property of the truncated protein and the specification discloses the complete sequence of the AAVRep78 protein. Therefore, the claimed "truncated AAVRep78 protein" is not indefinite to the skilled person because the claim and the specification provides an unambiguous definition of these truncated proteins.

Claims 14, 15, 17 and 18 are rejected as being indefinite for the lack of disclosure for the specific sequence for the tat protein of HIV and/or not providing a reference a reference of the specific sequence. On page 14, lines 27-28 of the specification, it is disclosed that a publication by Nagahara *et al.* discloses the use of the tat protein in fusion proteins. Also attached is an HIV Sequence Database provided by the U.S. government (Attachment 10) showing tat alignments on line. Additionally, a search of the NCBI Protein sequence database shows almost 1700 hits of the tat protein (Attachment 11). Applicant submits that tat protein of HIV is well known and available to skilled persons, and it is requested that this rejection be withdrawn.

CONCLUSION

Applicant believes that the present application is now in condition for allowance. Favorable reconsideration of the application as amended is respectfully requested.

The Examiner is invited to contact the undersigned by telephone if it is felt that a telephone interview would advance the prosecution of the present application.

The Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 19-0741. If any extensions of time are needed for timely acceptance of papers submitted herewith, Applicant hereby petitions for such extension under 37 C.F.R. §1.136 and authorizes payment of any such extensions fees to Deposit Account No. 19-0741.

Respectfully submitted,

Date January 21, 2004

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Avigen Inc. Press Releases

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Avigen Reports First Promising Human Gene Therapy Trial Results for Hemophilia B

Researcher from The Children's Hospital of Philadelphia Provides Update at The American Society of Hematology Meeting

NEW ORLEANS -- December 6, 1999 - Presenting at the annual meeting of the American Society of Hematology, researchers at Avigen (Nasdaq; AVGN), The Children's Hospital of Philadelphia, and Stanford University Medical Center reported that Avigen's gene therapy for treating hemophilia B is howing early indications of therapeutic benefit. Patients with hemophilia B are deficient in a protein called factor IX that is required for blood to clot. After delivering the gene for factor IX using Avigen's proprietary adeno-associated vector (AAV), factor IX protein activity was detected in the blood of the initial hemophilia patients treated, even at the lowest dose administered. "What we have observed is that the gene has been successfully delivered and is producing biologically active factor IX," said Avigen President and CEO, John Monahan, Ph.D. "Over the last several months, we were pleased to observe that the increase in factor IX protein activity correlated with a significant reduction of factor IX concentrate usage by these patients. This is a result we would not have anticipated at this dose level," added Dr. Monahan. Muscle biopsies have also confirmed the presence of factor IX in the tissue of patients who received the AAV vector. The trial began approximately six months ago. The initial patients began to express factor IX within eight weeks of the treatment and continue to express factor IX.

Three patients have now been treated using Avigen's proprietary adeno-associated vector (AAV) and have experienced no adverse side effects. No antibody formation against factor IX or localized inflammation at the injection site was seen in any of the patients during the course of the study. The results of the initial low dose regimen suggest that the treatment is safe and easy to administer. Treatment of patients at a higher dose level has recently begun.

Hemophilia B is a disease afflicting approximately 5,000 individuals in the U.S. Patients with hemophilia B lack clotting factor IX and must undergo intravenous infusions of factor IX protein concentrates at an annual cost of up to $100,000 per patient. The protein infusions are effective at providing temporary relief from acute bleeding episodes, but last only a few days in the circulation. Due to the lack of sustained levels of factor IX, patients with hemophilia experience frequent spontaneous bleeding episodes, particularly in their joints and soft tissues. With time, these patients generally develop chronic arthropathy or joint dysfunction. A small percentage suffer permanent disability or even die from bleeds into the central nervous system.

Coagulin B is Avigen's proprietary AAV vector carrying the gene for factor IX. Avigen's goal is to deliver the gene for factor IX into the patient's muscle cells wher it will continuously produce factor IX in the blood. Sustained levels of factor IX in the blood stream is expected to substantially reduce spontaneous bl eding episodes and the need for factor IX protein infusion. Nine patients are to be enrolled in the trial: three each at a low, interm diate and high dose, treated sequentially. In all cases, the treatment is a

simple procedur in which a single intramuscular administration delivers the vector containing th factor IX gene. The trial is b ing conducted at two sites: The Children's Hospital of Philadelphia, with lead investigators Katherine High, M.D. and Catherine Manno, M.D., and Stanford University Medical Center, where Mark Kay, M.D., Ph.D. and Bert Glad r, M.D., Ph.D. are leading the trial.

"The absence to date of any adverse reactions or side effects reaffirms Avigen's choice of our AAV vector system as a preferred vehicle for the delivery of genes in a safe and efficient manner. It is still early in the trial and we have a lot of work ahead of us, but we are very pleased by the results we have seen to date." Monahan added.

Based in the San Francisco Bay area, Avigen, Inc., is a biotechnology company involved in the development of gene therapy products derived from AAV for the treatment of inherited and acquired diseases. The company's proposed gene therapy products are designed for in vivo administration to achieve the production of therapeutic proteins within the body. Additional information on Avigen's proprietary gene therapies can be found at www.avigen.com

Note: Except for the historical information contained herein, this news release contains forward-looking statements that involve risks and uncertainties. Actual results regarding the efficacy of gene therapy using AAV vectors for treating humans with hemophilia B may differ materially from those discussed herein. Factors that could cause or contribute to such differences include, but are not limited to: the risk that the results reported are a statistical anomaly, and actual results with more data points will show less favorable results; the study is in its early phase, and the benefits experienced to date could drop off substantially over time; the risk that adverse side effects could still manifest themselves; the other factors discussed in this press release; and other risks detailed from time to time in documents filed by Avigen with the SEC, including the report on Form 10-K for the year ended June 30, 1999.

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Success of Gene Therapy Research for Canine Hemophilia B Shows Promise for Treating Humans

Research Results Published This Week in Nature Medicine

PHILADELPHIA (January 4, 1999) - Gene therapy using the adeno-associated virus (AAV) vector to deliver the missing clotting factor in hemophilia B "is likely to be successful in the treatment of humans with the disorder," according to research reported this week in the January 1999 issue of Nature Medicine. The report cites successful expression of biologically active clotting Factor IX in dogs that received a single administration of the gene therapy up to 16 months earlier.

"The gene transfer strategy that we used resulted in sustained expression of Factor IX at levels adequate to show improvement in two different blood coagulation assays," said Katherine High, M.D., Director of Research of the Hematology Division at The Children's Hospital of Philadelphia. "Many gene transfer strategies that seemed promising in mice failed to show efficacy in large-animal models. However, our success with large animal models here increases our expectation that similar effects may be seen in humans," she explained.

"The success of this simple, safe and efficient procedure for gene transfer and expression of Factor IX in a large-animal model also indicates potential applications for treatment of other genetic disorders that require systemic delivery of a therapeutic protein," the authors wrote. The research was conducted by a team of scientists from The Children's Hospital of Philadelphia, the University of North Carolina at Chapel Hill, and Avigen, Inc., (NASDAQ: AVGN) of Alameda, Calif. Dr. High is a member of Avigen's Scientific Advisory Board.

Avigen is a biotechnology company whose proprietary technology uses an AAV vector, a modified form of a common, harmless virus, to deliver genetic material to target cells that will then produce the missing protein implicated in inherited and acquired genetic disorders. An Investigational New Drug (IND) application was filed with the FDA in November 1998, seeking approval of this gene therapy approach for treating hemophilia B in humans.

Hemophilia B is a bleeding disorder caused by the absence or deficiency of clotting Factor IX that affects an estimated 2,800 U.S. males. Now, patients must undergo costly intravenous infusions of blood clotting Factor IX protein. The protein infusions are effective at correcting bleeding episodes, but last for less than 24 hours in the circulation. Annual treatment costs are estimated to be $100,000 per patient. For those reasons, patients are usually treated only in response to bleeding episodes. Micro-bleeds may continue to occur, causing damage to the joints or, in the worst case, a stroke due to bleeding in the brain.

The report in Nature Medicine also cites evidence that the Factor IX in the treated animals is biologically active; the animals' clotting times were significantly reduced. The report

notes that a period of 6 to 8 weeks was required to reach maximum levels of g ne expression and correction of clotting times, and that, subsequ ntly, expression lev ls have remained stable for more than a year after the one-time dose.

Further, none of the dogs developed immune responses that would limit long-term systemic expression of Factor IX. While researchers detected evidence of antibody response in some of the dogs using highly sophisticated techniques, they pointed out that if testing for antibody response had been confined to the measurements now in use in clinical settings, "all dogs currently on study would be deemed free of clinically significant antibody response," stated Dr. High. There was no evidence of local or systemic toxicity in the treated dogs.

The authors also note that the therapy was delivered via minimally invasive intramuscular (IM) injections. Although Factor IX is normally produced in the liver, these findings show that muscle cells can synthesize biologically active Factor IX.

In the same issue of Nature Medicine, Mark Kay, M.D., Ph.D., a current member of Avigen's Scientific Advisory Board, reported on a different research project in which gene therapy with recombinant AAV vectors corrected hemophilia B for a period of over 17 months in mice and for at least 8 months in dogs. Director of the Program in Human Gene Therapy and Associate Professor in the Departments of Pediatrics and Genetics at Stanford University School of Medicine, Dr. Kay is actively collaborating with Avigen as the company continues to expand its gene therapy research and development programs for the treatment of hemophilia A and B.

Based in the San Francisco Bay Area, Avigen, Inc., is a biotechnology company involved in the development of gene therapy products derived from AAV for the treatment of inherited and acquired diseases. The company's proposed gene therapy products are designed for in vivo administration to achieve the production of therapeutic proteins within the body.

Note: Except for the historical information contained herein, this news release contains forward-looking statements that involve risks and uncertainties. Actual results regarding efficacy of gene therapy using AAV vectors for treating humans with hemophilia B may differ materially from those discussed herein. Factors that could cause or contribute to such differences include, but are not limited to, those discussed in this press release, as well as other risks detailed from time to time in documents filed by Avigen with the SEC, including the report on Form 10-K for the year ended June 30, 1998. In particular, the statements above regarding the potential for gene therapy using AAV for treating humans with hemophilia B or other genetic disorders is subject, in addition to the above, to the following risks and uncertainties: success of gene therapy in large animal models does not guarantee that the same results will be obtained with humans; the risks of adverse side effects in humans; and other risks and uncertainties inherent in the development of gene therapy products.

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Avigen Reports In Vivo Delivery and Long-Term Expression of Factor VIII Gene For Hemophilia A

Alameda, Calif. (Nov. 1, 1999) In a gene therapy study targeting the potential treatment of hemophilia A, Avigen (NASDAQ: AVGN) scientists today announced that a single administration of an adeno-associated vector (AAV) carrying the gene for factor VIII produced physiological levels of biologically active clotting factor in the plasma of animals. Significant levels of factor VIII have persisted in these animals for nearly two years. Avigen believes this is the first publication showing factor VIII expression in vivo using AAV. "This is an important milestone in the development of AAV vectors to treat hemophilia A, indicating for the first time that AAV vectors represent a viable approach for factor VIII gene therapy," said Alan McClelland, Avigen's Vice President of Research and Development. This work parallels Avigen's clinical work with Coagulin-B®, an AAV vector carrying the gene for factor IX for treating hemophilia B which is currently in Phase I/II clinical trials and suggests that factor VIII can similarly be delivered using AAV for the treatment of hemophilia A. The findings were published in the October 26, 1999 issue of the Proceedings of the National Academy of Sciences.

"By successfully delivering a very large gene, once again our scientists have overcome what had been perceived as an obstacle," noted Avigen President and CEO John Monahan, Ph.D. "By splitting the human Factor VIII gene and delivering the two sequences via two separate AAV vectors, they have overcome the size limitations of the AAV vector. This certainly bodes well for our hemophilia A program."
This study is one component of the preclinical research underway on Avigen's proposed gene therapy product, Coagulin-A to treat hemophilia A. The program also includes approaches intended to deliver the factor VIII gene in a single AAV vector. Hemophilia A is an inherited disorder characterized by a deficiency in the blood clotting protein factor VIII. It affects roughly 15,000-20,000 individuals in the U.S. alone; the annual cost of treating these patients with recombinant or plasma-derived factor VIII exceeds $1.2 billion.
Avigen's factor IX gene therapy product for hemophilia B, Coagulin-B®, is currently in clinical trials at The Children's Hospital of Philadelphia and Stanford University Medical Center. The clinical protocol is designed to deliver an AAV vector containing the factor IX gene to hemophiliacs by means of an intramuscular injection
Based in the San Francisco Bay area, Avigen, Inc. is a biotechnology company involved in the development of gene therapy products derived from adeno-associated virus for the treatment of inherited and acquired diseases. The Company's proposed gene therapy products are designed for in vivo administration to achieve the production of therapeutic proteins within the body.
Citation:- Melissa Burton, Hiroyuki Nakai, Peter Colosi, Janet Cunningham, Rachel Mitchell, and Linda Couto. Coexpression of factor VIII heavy and light chain adeno-associated viral vectors produces biologically active protein. Proceedings of the National Academy of Sciences 1999; 96(22):12725-12730.
Note: Except for the historical information contained herein, this news release contains forward-looking statem nts that involve risks and uncertainti s. Actual results may diff r materially from those discussed herein. Factors that could cause or contribute to such differences include, but ar not limited to, those discussed in this press release, as well as oth r risks d tailed from time to time in documents filed by Avigen with the SEC, including

its Annual Report on Form 10-K for the year ended Jun 30, 1999. In particular, the foregoing discussion of the success of Avigen's delivery of Factor VIII in animal studies does not guarantee that the same results will be obtained with humans. In addition, other risks and uncertainties inh rent in the development of gene th rapy products may prevent Avigen's success in animal models from being r plicated in humans.

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Avigen's Gene Therapy Technology Shows Promise For Treating Parkinson's Disease

Research Presented by Dr. Krys Bankiewicz at The American Society of Gene Therapy Meeting Holds Promise For Novel Therapeutic Approach to Treating Common Neurological Disease

WASHINGTON, DC – June 14, 1999 – Krys Bankiewicz, M.D., Ph.D., of the National Institutes of Health, in collaboration with Avigen, Inc. (NASDAQ: AVGN) and Lawrence Berkeley National Laboratory presented research results of studies in primates suggesting the potential for Avigen's gene therapy technology to treat Parkinson's disease. Parkinson's disease is a neurological disorder characterized by a decrease in spontaneous movements, gait difficulty, postural instability, rigidity and tremor, which result from a decrease in the availability of the neuro-transmitter dopamine in the brain. Speaking at The American Society of Gene Therapy Conference in Washington, DC, Dr. Bankiewicz announced that dopamine activity could be restored in a primate model of Parkinson's disease following treatment with Avigen's adeno associated virus (AAV) vector.

"Researchers in the field of gene therapy have encountered many obstacles in their attempt to develop an effective therapeutic approach to treating genetic disorders," commented Dr. Bankiewicz. "Avigen's AAV based approach to treating Parkinson's disease may hold promise for the broader application of treating a variety of genetically based conditions," he said. Dr. Bankiewicz's findings represent an important advance in the potential development of gene therapy treatment for Parkinson's disease, a common and devastating neuro-degenerative disease affecting over one million people in the United States.

The strategy behind gene therapy is to provide the patient with the genetic information needed in order to restore certain biological activities within the cell. The challenge to date has been to identify a mechanism by which genetic information can be successfully transferred to the appropriate cells to the body. Viruses have been identified as possible delivery agents; however, many viruses are harmful or even deadly and are effective only under certain carefully controlled conditions. As a result of its progress with its AAV vector system, Avigen researchers may have developed a potential solution to this challenge.

"We are enthusiastic about the possible application of Avigen's gene therapy technology to Parkinson's disease," said John Monahan, Ph.D., President and CEO of Avigen. "If our research findings continue to exhibit the promise which they have to date, our AAV vector system may provide an attractive therapeutic alternative for certain genetically based diseases."

Ba ed in the San Francisco Bay Area, Avigen, Inc. is a biot chnology company involved in th development of gene th rapy products derived from adeno-associated virus for the treatment of inherited and acquired diseases. The Company's proposed gene therapy products are designed for in vivo administration to achiev the production of therapeutic

proteins within the body. Avigen recently commenced a Phase I human clinical trial of Coagulin-B®, its new gene therapy treatment for hemophilia B.

Ernest Orlando Lawrence Berkeley National Laboratory (Berkeley Lab) engages in non-classified scientific research in Berkeley, CA. It is managed by the University of California for the U.S. Department of Energy.

Note: Except for the historical information contained herein, this news release contains forward looking statements that involve risks and uncertainties. The statements regarding the therapeutic value of AAV gene therapy for the treatment of Parkinson's disease, the therapeutic value of Coagulin-B® for the treatment of Hemophilia B and estimates of the numbers of patients that are potential candidates for these treatments, are forward-looking statements subject to risks and uncertainties, including: the success of gene therapy and administration in animal models does not guarantee that the same results will be obtained in humans; the risk that side effects may result from the treatments; other risks and uncertainties inherent in the development of gene therapy products; and those risks detailed from time to time in documents filed by Avigen with the SEC, including the report on Form 10-K for the year ended June 30, 1998.

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Comparison of the *in vitro* transforming activities of human papillomavirus types

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Communicated by L.Crawford

The association of certain human papillomavirus (HPV) types with the majority of human cervical carcinomas suggests a role for the virus in the development of this type of cancer. In this paper, we have examined the transforming properties of several HPV types where the early region genes of the virus are under the control of a strong heterologous promoter and show that major differences exist between the HPV types in their ability to transform primary rat kidney epithelial cells in conjunction with an activated *ras* oncogene. Those HPV types most commonly found in carcinomas—types 16, 18, 31 and 33—are capable of co-operating with *ras* to transform primary cells, but those types most commonly found in benign lesions—types 6 and 11—are not. We further demonstrate that the E7 gene of HPV16 by itself is sufficient to co-operate with activated *ras* to produce transformed cells which are tumorigenic in immunocompetent animals.

Key words: transformation/human papillomavirus/E6/E7

Introduction

One of the most striking differences in the natural history of the human genital papillomavirus infection is in the prognosis of lesions containing human papillomavirus (HPV) type 6 or 11 DNA on the one hand and those containing HPV16, 18, 31 and 33 DNA on the other. HPV6 and HPV11 DNAs are frequently found in genital warts and mild dysplasias of the uterine cervix, but rarely in severe dysplasias or malignant carcinomas. The other types—16, 18, 31 and 33—are rarely found in condylomas or mild dysplasias, but their DNAs are found in most severe dysplasias and malignant carcinomas (Dürst *et al.*, 1983; Gissman *et al.*, 1983).

This suggests very strongly that there must be some significant differences between the genes of the various types in the way that infected cells respond to them. HPV6 and HPV11 are relatively closely related, showing >60% homology throughout their genomes (Broker and Chow, 1986). HPV6 is less closely related to HPV18 and types 16 and 18 are not closely related to each other (Boshart *et al.*, 1984). The closest homology between HPV types is found in the L1 open reading frame, corresponding to the major capsid protein (Broker and Chow, 1986) but this is unlikely to be involved in cellular transformation. The different probabilities that HPV types exhibit of inducing malignant transformation and carcinomas, as opposed to the production of benign papillomas, suggests that DNA sequence differences exist between the various HPV types which are important in determining the transforming potential of an HPV type.

In this paper we have used an *in vitro* co-transformation assay (Matlashewski *et al.*, 1987a) to examine the biological activities of HPV16, HPV18, HPV31, HPV33, HPV6 and HPV11. The advantages of this co-transformation assay using primary baby rat kidney (BRK) epithelial cells are threefold. Firstly, the cells are epithelial rather than fibroblastic and, since true papillomaviruses *in vivo* grow only in epithelial cells, this is a much more appropriate cell type. Secondly, the cultures are primary cells rather than established cell lines and are therefore presumed to be normal when placed in culture. Thirdly, the system shows a requirement for two oncogenes or transforming agents, one from the establishment class and one from the transforming class. It is similar in this respect to the primary rat cell system which has been studied extensively (Land *et al.*, 1983; Rassoulzadegan *et al.*, 1983; Ruley, 1983). The requirement for more than one transforming event parallels the situation *in vivo*, where it is clear that papillomavirus infection alone is not sufficient to generate malignantly transformed cells. A second agent such as smoking, X-rays or chemical carcinogen is probably needed to allow the development of a high-grade lesion into a carcinoma. In our previous study we showed that, in conjunction with an activated *ras* oncogene, the DNA of HPV16 under control of a strong heterologous promoter transformed primary rat kidney epithelial cells. This activity requires a protein or proteins from the E6/E7 region of the HPV16 genome. We have now refined this localization and show that the relevant protein is likely to be E7. We then went on to examine other papillomavirus DNAs and show that there are striking differences between the various HPV types in their transforming activity.

Results

E7 is the transforming gene of HPV16

Transfection experiments using HPV16 DNA cloned into a pZIP-NeoSV(X)1 vector (Cepko *et al.*, 1984) forming the plasmids HZIP-16 and HPV16K (Matlashewski *et al.*, 1987a), demonstrated that HPV16 contains one or more oncogenes, derived from the E6/E7 region of the genome, which could co-operate with an activated *ras* gene to transform primary cells (Matlashewski *et al.*, 1987a). We first set up experiments to determine whether the transforming activity resides in E6 or E7 or whether both were required. Using an expression vector similar to pZIP-Neo, pJ4Ω (Wilkinson *et al.*, 1988), we cloned the E6 or E7 open reading frames (ORFs), either separately or together, and tested their transforming ability relative to pJ4Ω16 which contains most of the HPV16 genome (Figure 1). The plasmid pJ4Ω16K contains both the E6 and E7 genes on a DNA fragment derived from the deletion mutant HPVK-16 (Matlashewski *et al.*, 1987a). A plasmid containing the E6

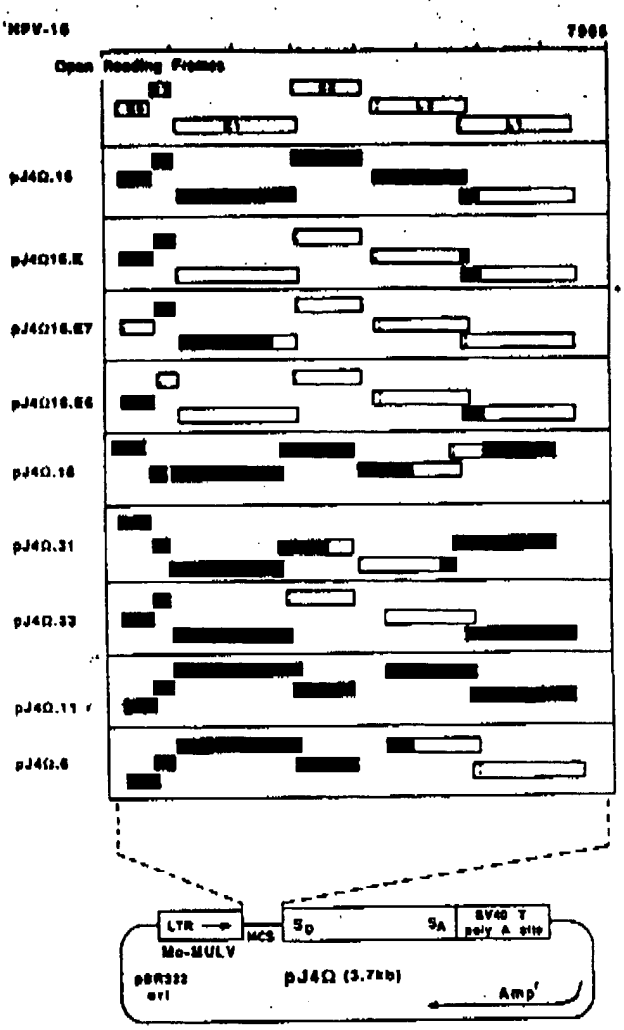

Fig. 1. Structure of recombinant HPV DNA containing plasmids. HPV ORFs present are indicated by solid bars. Mo-MuLV LTR, ampicillin resistance marker, multiple cloning sites (MCS) and splice donor (SD) and splice acceptor (SA) sites are indicated. The diagram is not to scale. The locations of the HPV ORFs are taken from Schwarz *et al.* (1983), (HPV6); Dartmann *et al.* (1986), (HPV11); Seedorf *et al.* (1985), (HPV16); Cole and Danos (1987), (HPV18); Lorincz *et al.* (1986), (HPV31); Beaudenon *et al.* (1986), (HPV33).

gene alone was constructed from a pAT153 vector (Twigg and Sherratt, 1980) containing HPV16 DNA inserted at the *Bam*HI site. This plasmid was deleted between the *Pvu*II site at bp 551 and the *Nco*I site at bp 863, and also between the two *Kpn*I sites at bp 880 and 5377. This deleted plasmid was restricted with *Eco*RI and *Bam*HI and a 1.77-kb fragment was inserted into pJ4Ω. This plasmid, however, contained the E6 ORF which was in the wrong orientation with respect to expression from the LTR promoter. The fragment was correctly orientated by cutting with *Bam*HI, end repairing, then cutting with *Eco*RI. The resulting E6 ORF containing fragment was inserted into a *Bgl*II-cut, end-repaired, *Eco*RI-cut pJ4Ω to form the fragment pJ4Ω16.E6 (Figure 1). This plasmid codes for the entire E6 ORF, except for the C-terminal amino acid leucine which is replaced by His-Gly followed by a stop codon. To generate the E7 vector, a 1.9-kb *Ns*II fragment containing the E7 ORF was linker adapted such that both *Ns*II sites were regenerated and both *Bam*HI and *Eco*RI sites were added at either end. This fragment was inserted into the *Eco*RI site of pAT153. This plasmid was restricted with *Bam*HI and the E7 fragment inserted into pJ4Ω to form the plasmid pJ4Ω16.E7. It contains the E7 ORF with virtually no upstream sequence and with 1.6 kb of 3' sequence beyond the E7 stop codon (Figure 1).

These HPV16 DNA containing plasmids were transfected into BRK cells with or without an activated EJ-*ras* gene. The plasmid pSV2-neo (Southern and Berg, 1982) was included in all transfection experiments. After 24 h the cells were placed under G418 selection. Transformation was monitored by the appearance of foci after 3 weeks (Figure 2). Neither pSV2-neo nor EJ-*ras* by themselves formed foci.

As shown in Table I, the activity of the plasmid pJ4Ω16K localizes the transforming activity of HPV16 to the E6/E7 region, confirming the previous result with HPVK-16 which contains the same sequences in the pZIP-NeoSV(X)1 vector. No foci were ever observed in experiments using pJ4Ω16.E6. However, when pJ4Ω16.E7 was co-transfected with EJ-*ras* the number of foci formed was about the same as that formed with pJ4Ω16. This shows that the E7 gene alone is capable of co-transforming primary cells. In conjunction with our previous results it shows that E7 is both necessary and sufficient for transformation in this assay. The cells produced by transfection with pJ4Ω16.E7 are morphologically altered, growing quickly to high density and showing no signs of senescence after >4 months. The numbers of foci obtained using pJ4Ω16K were always lower than those obtained when either pJ4Ω16 or pJ4Ω16.E7 are used (Table I). However, when pJ4Ω16.E6 and pJ4Ω16.E7 were transfected together, similar numbers of foci to those generated by pJ4Ω16 were observed. This suggests that the lower number of foci formed by pJ4Ω16K is a function of that particular construct, and that the E6 gene does not inhibit transformation by E7. It is possible that a spliced E6−E7 product from the pJ4Ω16K insert has a negative effect on transformation and that this negative effect is lacking where E6 and E7 are on separate plasmids. To test the transforming activities of E7 genes from HPVs 18, 31 and 33, E7 genes from these types were isolated and cloned into pJ4Ω. For HPV18, a 1.4-kb *Xba*I fragment (bp 321−1735), for HPV31, a 2.0-kb *Hpa*I−*Eco*RV fragment and for HPV33 a 644-bp *Dra*I fragment (bp 470−1114) were used. Each of these E7 alone constructs co-operated with *ras* as efficiently as constructs containing the entire early region.

The transformed cells from colonies of the pJ4Ω16 and pJ4Ω16.E7 transfections were expanded and analysed for HPV16 DNA by Southern blot analysis (Southern, 1975). Total cellular DNA from these cell lines was digested with *Bam*HI and probed with nick-translated HPV16 DNA. Figure 3 shows that integrated HPV16 DNA was present at high copy number in the co-transfected cell lines. Northern blot analysis of total RNA isolated from transiently infected BRK cells shows that HPV16 early region RNA species were expressed (Figure 4a). These BRK cells express RNA species which range in size from ~2 kb to ~5 kb in length. Western blot analysis of a pJ4Ω16-transformed monoclonal cell line demonstrated the presence of E7 protein in these cells (Figure 4b). Immunoperoxidase staining of either pJ4Ω16- or pJ4Ω16.E7-transformed cells using a monoclonal antibody directed against v-H-*ras* showed overexpression of *ras* in these cells (data not shown).

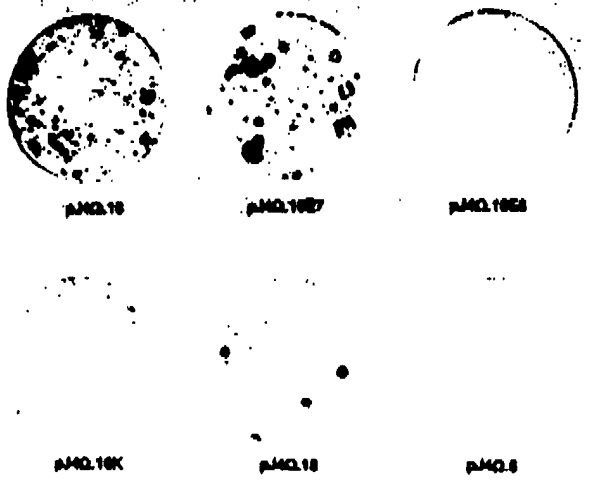

Fig. 2. Transformation of primary BRK cells by HPV DNA. All dishes were transfected with pSV2-neo, pEJ6.6 (containing an activated *ras* oncogene) and the indicated plasmid in each case. Cultures were grown in the presence of 200 μg/ml G418 and stained 3 weeks after transfection.

Table I. Number of foci obtained from plasmid treatment of BRK cells

Plasmid	Experiment		
	1	2	3
pJ4Ω	34	116	38
pJ4Ω16K	7	62	11
pJ4Ω16.E7	15	130	63
pJ4Ω16.E6	0	0	0
pJ4Ω16.E7+pJ4Ω16.E6	24	133	37
pJ4Ω18	17	51	44
pJ4Ω31	16	42	52
pJ4Ω33	27	40	31
pJ4Ω16+pJ4Ω6	30	141	35
pJ4Ω16+pJ4Ω11	27	156	77
pJ4Ω6	0	0	0
pJ4Ω11	0	0	0
pJ4Ω vector alone	0	0	0

Each experiment contained, in addition to the indicated plasmid, pSV2-neo encoding resistance to G418 and pEJ6.6, a plasmid containing the Ha-*ras* oncogene derived from the human bladder carcinoma cell line. The number of foci are those obtained when 5 μg of each plasmid was added to a 90-mm dish of primary sub-confluent BRK cells. No foci were observed if either pSV2-neo or pEJ6.6 were omitted from the experiment.

Co-transformation with other HPV types

To investigate the co-transforming potential of other HPV types, DNA fragments encoding early region ORFs were ligated into pJ4Ω (Figure 1). Constructs were modelled on HZIP-16 as far as possible and contained analogous sequences. HPV6 inserted at the *Bam*HI site of pAT153 was cut with *Bam*HI and *Mlu*I. A 5.2-kb fragment encoding early region genes was isolated and end repaired. This fragment was *Bam*HI linked and cloned into *Bam*HI-cut pJ4Ω. The plasmid pJ4Ω11 was constructed by excising the HPV11 genome with *Bam*HI from the pSV2-neo vector and inserting this fragment into pJ4Ω. The HPV18 genome in pBR322 was cut out with *Eco*RI and recircularized using T4 DNA ligase. The circularized genome was digested with *Kpn*I and a 6.4-kb fragment inserted into pJ4Ω. HPV31 DNA, inserted at the *Eco*RI site of pBR322, was cut with *Eco*RI and *Sal*I, yielding a 5.8-kb fragment which was ligated into *Eco*RI/*Sal*I-cut pJ4Ω. HPV33 DNA present in a *Bgl*II site of a modified pBR322 vector was cut with *Hin*dIII and *Bgl*II, giving a 5.6-kb fragment which was cloned into *Hin*dIII/*Bgl*II-cut pJ4Ω. These fragments were transfected into BRK cells as described and placed under G418 selection. The results of these experiments are summarized in Table I. The vectors containing DNA from HPV types 16, 18, 31 and 33 produced similar numbers of transformed foci, whereas no foci were observed with HPV6 or HPV11 constructs. This could have been either because they do not express their early regions or because the proteins expressed lacked transforming activity. To verify that the HPV6 and 11 constructs were expressing early region RNA, BRK cells were transiently infected with pJ4Ω6 and pJ4Ω11. Total cellular RNA was probed with virus-specific early region DNA and compared to RNA isolated from pJ4Ω16-, pJ4Ω16.E7- and pJ4Ω18-transformed BRK cells (Figure 4a). Similar levels of early region transcript were seen in the cells transfected with HPV6, 11 or 16, suggesting that the lack of transforming activity in HPV6 and 11 is not simply due to the absence of early region expression. Furthermore, isolated E7 ORFs

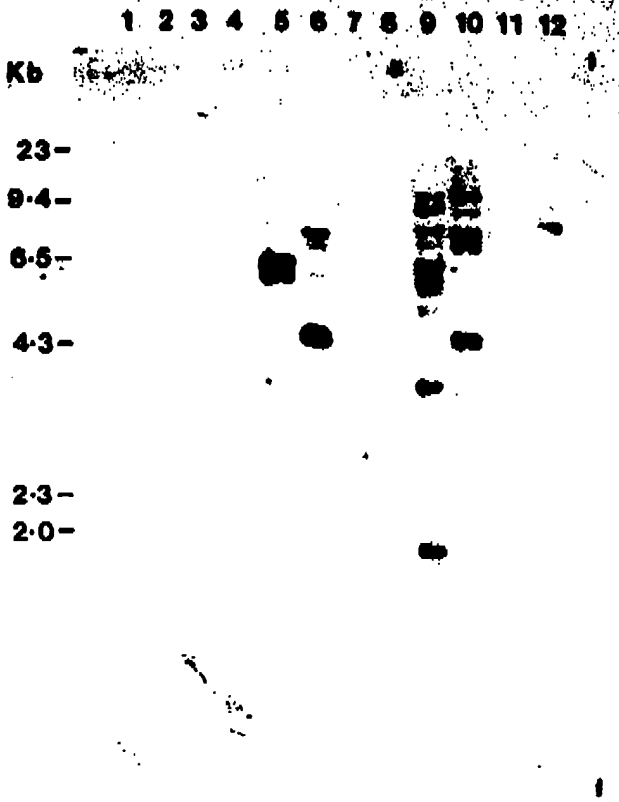

Fig. 3. Southern blot analysis of HPV16 DNA sequences in BRK cells which had been co-transfected with HPV16 DNA, pSV2-neo and the EJ-*ras* oncogene. Each lane contains 10 μg genomic DNA. Lanes 1–3 contain pJ4Ω digested with *Bam*HI, *Kpn*I and *Pst*I. Lanes 4–7 contain DNA obtained from a monoclonal cell line derived from a single transformed foci containing pJ4Ω16 (lane 4, uncut DNA; lanes 5–7 are digested with *Bam*HI, *Kpn*I and *Pst*I). Lanes 8–11 contain DNA isolated from a monoclonal cell line transformed with pJ4Ω16.E7 (lane 8, uncut DNA; lanes 9–11 contain DNA cut with *Bam*HI, *Kpn*I and *Pst*I). Lane 12 contains 30 pg *Bam*HI cut HPV16 DNA, equivalent to one copy of HPV DNA per cell.

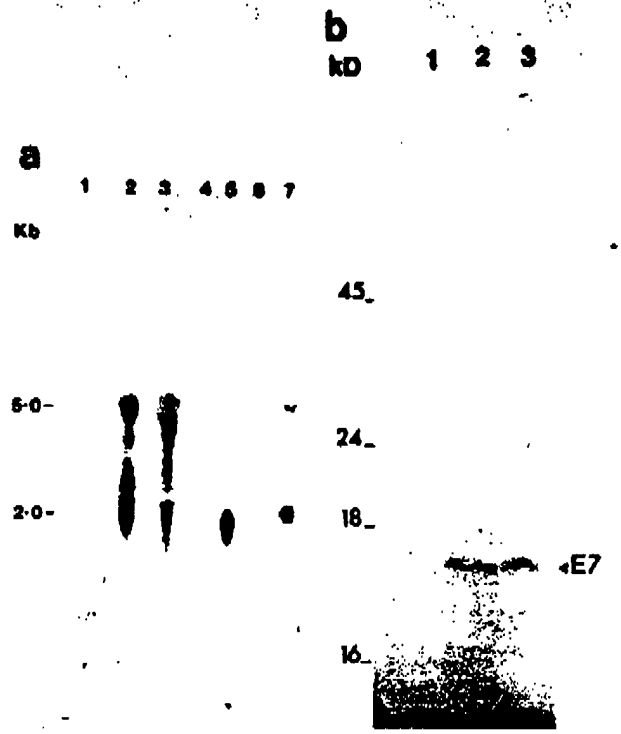

Fig. 4. (a) Northern blot analysis of RNA derived from transiently infected BRK cells. Lane 1, pJ406; lane 2, pJ4Ω11; lane 3, pJ4Ω18; lane 4, pJ4Ω16; lane 5, pJ4Ω16.E7; lane 6, uninfected BRK cells; lane 7, HT156, a tumour cell line containing HPV18 DNA. Each lane was probed with a DNA probe corresponding to the regulatory and E6/E7 regions specific for the corresponding HPV type. The probes were: HPV6, a 2.75-kb *Hin*dIII fragment derived from pJ406; HPV11, a 2.25-kb *Bam*HI–*Sph*I fragment of genome DNA; HPV16, the 2.1-kb *Bam*HI insert of pJ4Ω16K (nucleotides 5377–6150 and 7454–880); HPV18, a 2.4-kb *Kpn*I–*Taq*I fragment of genome DNA. (b) Western blot analysis. Cell extracts of: lane 1, untransfected BRK cells; lane 2, CaSki cells; lane 3, pJ4Ω16 cells were run on a 12% polyacrylamide gel and the proteins transferred to a nitrocellulose filter. The filter was then reacted with the anti-E7 monoclonal antibody E7IV (Ottersdorf *et al.*, 1987) followed by immunoperoxidase detection.

from HPV types 18, 31 and 33 are able to co-operate with *ras* as efficiently as the entire early region of the respective genomes to transform primary BRK cells.

Tumour production

Acquisition of a transformed phenotype *in vitro* is by no means always accompanied by increase in tumorigenic potential. We therefore cloned and tested the cell lines generated by transformation with HPV DNA plus *ras* for their ability to produce tumours in syngeneic rats. When 2×10^6 cells transformed by either pJ4Ω16 or pJ4Ω16.E7 were injected subcutaneously into each flank of three syngeneic rats tumours formed at the site of injection 2–3 weeks later. These tumours were found to be poorly differentiated adenocarcinomas.

Cytokeratin staining

Since the tumours with which HPV types are associated are usually carcinomas, derived from epithelial cells, it was important to show that the cells or cell lines generated by *in vitro* transformation were epithelial in origin rather than fibroblastic. By using specific monoclonal antibodies directed against the cytokeratins characteristic of epithelial cells we were abl to demonstrate that the transformed cells were,

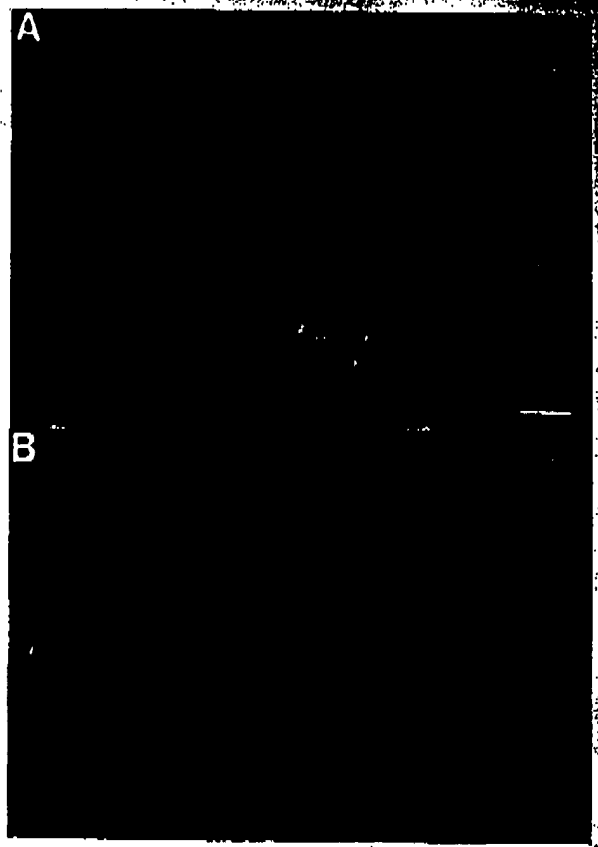

Fig. 5. Immunofluorescence patterns of monoclonal antibody (mAB) staining on methanol fixed BRK cells transformed with pJ4Ω16.E7. Panel A was stained with mAB LE61 (keratin 18) and B with mAB LP34 (keratins 5 and 14). Scale bar = 2.5 μm.

in all cases, simple epithelial cells. They were stained with monoclonal antibody LE61, specific for keratin 18 (Figure 5). No staining was observed with LP34, which in rat cells stains keratins 5 and 14 but not simple keratins such as keratin 18 (Figure 5) (Lane *et al.*, 1985).

Discussion

The best-characterized *in vitro* transformation system is the one developed for bovine papillomavirus type 1 (BPV-1). The BPV-1 genome contains at least two separate transforming genes, E5 and E6 (Nishibayashi *et al.*, 1983; Sarver *et al.*, 1984; Schiller *et al.*, 1984; Yang *et al.*, 1985; DiMaio *et al.*, 1986). Transformation of mouse fibroblast cell lines by HPV16 was obtained more recently, using assay systems similar to those used for BPV-1 (Tsunokawa *et al.*, 1986; Yasumoto *et al.*, 1986; Matlashewski *et al.*, 1987b). Subsequently transformation of primary rat epithelial cells was achieved by using HPV16 in conjunction with an activated *ras* oncogene (Matlashewski *et al.*, 1987a). Here deletion analysis indicated that the transforming protein or proteins encoded by HPV16 came from the E6/E7 region of the genome and that none of the other major ORFs was required (Matlashewski *et al.*, 1987a).

We have now shown that the E7 gene by itself co-operates with an activated *ras* gene to transform primary BRK cells. Our conclusion, that HPV16-E7 is both necessary and sufficient for transformation in this assay, is surprising in view

of the results previously obtained with BPV-1. The analysis of BPV-1 transformation which defined E5 and E6 as transforming proteins did not seem to implicate E7. Its function would appear to be related to control of copy number when BPV-1 DNA is replicating episomally in mouse cells, although cells transformed by an E7 mutant were sometimes unstable (Lusky and Botchan, 1985; Berg *et al.*, 1986).

We had expected to find that HPV16-E6 had transforming activity analogous to that of BPV1-E6 and our results with the E6/E7-containing construct HPVK-16 were consistent with a role for E6 (Matlashewski *et al.*, 1987a). However, repeated efforts to obtain transformation with HPV16-E6 in pJ4Ω were uniformly negative, whereas HPV16-E7 always gave transformation. The E7 transforming activity was not antagonized by E6, nor did adding E6 cause a significant increase in E7 transformation. Complex splicing patterns have been observed for the E6/E7 region, generating RNAs which could code for polypeptides made up of parts of E6 and E7 ORFs (Schwarz *et al.*, 1985; Schneider-Gädicke and Schwarz, 1986). We do not have any evidence for a role of any protein derived from, or including part of, the E6 region in our transformation assay, but clearly in other circumstances, e.g. in the absence of a strong heterologous promoter, it may be important to influence the amount of E7 production. The difference between BPV-1 and HPV16 transformation may be related to the use of fibroblasts and epithelial cells respectively in the two assays, or more likely to the fact that co-transformation by HPV16 plus *ras* may relate to carcinomatous transformation as discussed later. Monoclonal cell lines transformed by pJ4Ω16.E7 plus *ras* or by pJ4Ω16 plus *ras* have been growing in culture for >4 months with no sign of senescence. These cells were clearly epithelial by their cytokeratin staining patterns and formed tumours in immunocompetent syngeneic rats within 2–3 weeks.

Having established that transfection with HPV16 plus *ras* generated malignantly transformed cells, we went on to ask whether other genital papillomavirus types were also active. The virus types tested fell into two distinct groups: types 18, 31 and 33, which were as active as HPV16; and types 6 and 11, which were inactive. HPV6 and 11 are usually found in benign genital and laryngeal lesions and generate papillomas. They appear rarely to progress to carcinomas. HPV18, 31 and 33, on the other hand, are found in advanced dysplasias, intra-epithelial neoplasias and carcinomas *in situ*. Our analyses of virus-specific RNA in the HPV6- and HPV11-transfected cells indicated that the E6/E7 region of the DNA was being transcribed, showing that viral genes are being expressed by these types, although transformation is not occurring. Transfection of HPV6 or HPV11 together with HPV16 caused no reduction in transformation by the active type, indicating that HPV6 and HPV11 do not have a negative effect in blocking transformation or killing transformed cells. The DNA sequences of HPV6, 11, 18, 31 and 33 are quite similar overall and in the E7 region in particular. It will be interesting to compare the properties of the viral E7 polypeptides to determine whether this is where critical differences reside and, if so, where within the E7 polypeptide they are located.

There is a striking parallel between the *in vivo* malignancy of HPV-associated lesions and the activity of the corresponding HPV type in our co-transformation assay. This strongly supports the idea that we are studying the carcinomatous type of transformation rather than simply the benign papillomatous type of transformation. This correlation provides a valuable means of investigating the role of papillomaviruses in the development of cervical cancer.

Materials and methods

Construction of HPV expression plasmids

HPV DNAs were kindly provided by Professor H.zur Hausen (HPVs 6, 11, 16, 18), Dr A.T.Lorincz (HPV31) and Dr G.Orth (HPV33). The plasmid pJ4Ω was a gift from J.Morgenstern. DNA fragments of the different HPV types were cloned into the multiple cloning site of pJ4Ω as described earlier.

Transfection and selection

Cultures of primary BRK cells were prepared and transfected by the DNA–calcium phosphate co-precipitation method (Wigler *et al.*, 1979). Aliquots of DNA–calcium phosphate precipitate (0.4 ml) containing 5 μg of each of the indicated plasmids was added to 90-mm dishes of sub-confluent primary BRK cells. After glycerol treatment the cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 200 μg/ml G418. Cells were fixed in formal saline and stained with Giemsa stain.

Nucleic acid hybridization

Total genomic DNA was isolated from transformed BRK colonies which had been transfected with HPV DNA and the EJ-*ras* oncogene. DNA samples (10 μg) were digested with *Bam*HI, *Pst*I and *Kpn*I and electrophoresed in 1% agarose gels and subjected to Southern blot analysis (Southern, 1975) using Hybond-N filters (Amersham International). Hybridizations were performed using ^{32}P nick-translated HPV genomic DNA for 20 h. Filters were washed in 2 × SSC containing 0.1% SDS (1 × SSC is 150 mM NaCl, 15 mM trisodium citrate, pH 7.0) at room temperature, and then in 0.2 × SSC containing 0.1% SDS at 55°C and exposed to Fuji RX X-ray film with screens.

Total RNA was isolated from transiently infected BRK cells using guanidium thiocyanate (Chirgwin *et al.*, 1979). Glyoxylated RNA (20 μg) was electrophoresed on 1% agarose gels and subjected to Northern blot analysis (Thomas, 1980). Hybridizations were performed as described above.

Immunoblotting

For analysis of E7 protein synthesized in cell lines a 90-mm plate of confluent cells was lysed using 200 μl of lysis buffer (250 mM NaCl, 0.1% NP-40, 50 mM Hepes, pH 7.0, 1% aprotinin).

SDS–PAGE, immunoblotting and protein detection were as previously described (Banks *et al.*, 1987) using an anti-E7 monoclonal antibody E7IV (Ohnsdorf *et al.*, 1987).

Acknowledgements

We would like to thank Dr F.Watt for help with immunofluorescent techniques, Dr B.Lane for the gift of monoclonal antibodies LE61 and LP34 and Dr L.Glasmann for antibody E7IV. HPV DNAs were generous gifts from Professor H.zur Hausen (HPVs 6, 11, 16, 18), Dr A.T.Lorincz (HPV31) and Dr G.Orth (HPV33). We would like to thank Clare Middlemiss for preparing the manuscript.

References

Banks,L., Spence,P., Androphy,E., Hubbert,N., Matlashewski,G., Murray, A. and Crawford,L. (1987) *J. Gen. Virol.*, 68, 1351–1359.

Beaudenon,S., Kremsdorf,D., Croissant,O., Jablonska,S., Wain-Hobson,S. and Orth,G. (1986) *Nature*, 321, 246–249.

Berg,L.J., Singh,K. and Botchan,M. (1986) *Mol. Cell. Biol.*, 6, 859–869.

Boshart,M., Gissmann,L., Ikenberg,A., Kleinheinz,W., Scheurlen,W. and zur Hausen,H. (1984) *EMBO J.*, 3, 1151–1157.

Broker,T.R. and Chow,L.T. (1986) *Cancer Cells*, 4, 589–594.

Copin,C.K., Roberts,B.E. and Mulligan,R.C. (1984) *Cell*, 37, 1053–1062.

Chirgwin,J., Przybyla,A.E., MacDonald,R.J. and Rutter,W.J. (1979) *Biochemistry (Wash.)*, 18, 5294–5299.

Cole,S.T. and Danos,O. (1987) *J. Mol. Biol.*, 193, 599–608.

Dartmann,K., Schwarz,E., Gissmann,L. and zur Hausen,H. (1986) *Virology*, 151, 124–130.

DiMaio,D., Guralski,D. and Schiller,J.T. (1986) *Proc. Natl. Acad. Sci. USA*, 83, 1797–1801.

Dürst,M., Gissmann,L., Ikenberg,H. and zur Hausen,H. (1983) *Proc. Natl. Acad. Sci. USA*, 80, 3812–3815.

Giesmann,L., Wolak,L., Rosberg,H., Koldovsky,V., Schwarz,H. and zur Hausen,H. (1983) *Proc. Natl. Acad. Sci. USA.*, **80**, 560–563.
Lund,H., Parada,L.F. and Weinberg,R.A. (1983) *Nature*, **304**, 596–602.
Lane,E.B., Bartek,J., Purkis,P.E. and Leigh,I.M. (1984) *Ann. N.Y. Acad. Sci.*, **455**, 241–248.
Lorincz,A.T., Lancaster,W.D. and Temple,G.F. (1986) *J. Virol.*, **58**, 225–229.
Lusky,M. and Botchan,M. (1985) *J. Virol.*, **53**, 955–965.
Matlashewski,G., Osborn,K., Murray,A., Banks,L. and Crawford,L. (1987b) In Steinberg,B.M., Brandsma,J.L. and Taichman,L.B. (eds), *Cancer Cells. Vol. 5. Papillomaviruses*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 195–199.
Matlashewski,G., Schneider,J., Banks,L., Jones,N., Murray,A. and Crawford,L. (1987a) *EMBO J.*, **6**, 1741–1746.
Nakabayashi,Y., Chattopadhyay,S.K. and Lowy,D.R. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 5832–5836.
Otterdorf,T., Seedorf,K., Rowekamp,W. and Giesmann,L., (1987) *J. Gen. Virol.*, **68**, 2933–2938.
Rassoulzadegan,M., Naghashfar,Z., Cowie,A., Carr,A., Grisoni,M., Kamen,R. and Cuzin,F. (1983) *Proc. Natl. Acad. Sci. USA.*, **80**, 4354–4358.
Ruley,H.E. (1983) *Nature*, **304**, 602–606.
Sarver,N., Rabson,M.S., Yang,Y.C., Byrne,J.C. and Howley,P.M. (1984) *J. Virol.*, **52**, 377–388.
Schiller,J.T., Vass,W.C. and Lowy,D. (1984) *Proc. Natl. Acad. Sci. USA.*, **81**, 7880–7884.
Schneider-Gädicke,A. and Schwarz,E. (1986) *EMBO J.*, **5**, 2285–2292.
Schwarz,E., Durst,M., Demankowski,C., Lattermann,O., Zech,R., Wolfsperger,E., Suhai,S. and zur Hausen,H. (1983) *EMBO J.*, **3**, 2341–2348.
Schwarz,E., Freese,U.K., Gissmann,L., Mayer,W., Roggenbuck,B., Stremlau,A. and zur Hausen,H. (1985) *Nature*, **314**, 111–113.
Seedorf,K., Krammer,G., Durst,M., Suhai,S. and Rowekamp,W.G. (1985) *Virology*, **45**, 181–185.
Southern,E.M. (1975) *J. Mol. Biol.*, **98**, 503–517.
Southern,P.J. and Berg,P. (1982) *J. Mol. Appl. Genet.*, **1**, 327–341.
Thomas,P.S. (1980) *Proc. Natl. Acad. Sci. USA.*, **77**, 5201–5205.
Tsunokawa,Y., Takebe,N., Kasamatsu,T., Terada,M. and Sugimura,T. (1986) *Proc. Natl. Acad. Sci. USA.*, **83**, 2200–2203.
Twigg,A.J. and Sherratt,D. (1980) *Nature*, **283**, 216–218.
Wigler,M., Pellicer,A., Silverstein,S., Axel,R., Urlaub,G. and Chasin,L. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 1373–1376.
Wilkinson,D., De Vries,R.R.P., Madrigal,J.A., Lock,C.B., Morgenstern, J.P., Trowsdale,J. and Altmann,D.M. (1988) *J. Exp. Med.*, **146**, in press.
Yang,Y.C., Okayama,H. and Howley,P. (1985) *Proc. Natl. Acad. Sci. USA.*, **82**, 1030–1034.
Yasumoto,S., Burkhardt,A., Doniger,J. and DiPaolo,J. (1986) *J. Virol.*, **57**, 572–577.

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Transforming activity of human papillomavirus type 16 DNA sequences in a cervical cancer

(malignant tumor/DNA virus/transformation/NIH 3T3 cells)

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ABSTRACT A genomic DNA sample from cervical cancer tissue, containing human papillomavirus (HPV) type 16, was found to induce malignant transformation of NIH 3T3 cells when it was tested by transfection assays using the calcium phosphate coprecipitation technique. The primary and secondary transformants contained the HPV type 16 DNA sequences and human specific *Alu* family sequences. To the best of our knowledge, it has not been reported previously that HPV type 16 DNA sequences in total genomic DNA from a cervical cancer have transforming activity.

There is increasing evidence that human papillomavirus (HPV) types 16 and 18 are involved in the development of cervical cancers (1–11): HPV type 16 or type 18 DNA sequences and their transcripts are frequently found in cervical cancer tissues and cell lines established from cervical cancers. However, the only information available on the transforming activity of these types of HPV is a report that a recombinant plasmid containing a head-to-tail HPV type 16 dimer could transform NIH 3T3 cells (12). We tested the genomic DNA from a human cervical cancer containing HPV type 16 for transforming activity on NIH 3T3 cells by transfection using the calcium phosphate coprecipitation technique. We found that this DNA sample transformed NIH 3T3 cells and that the transforming activity was associated with the HPV type 16 DNA sequences and human specific *Alu* family sequences.

MATERIALS AND METHODS

Cells. NIH 3T3 cells and ~~al-1~~ cells were kindly provided by M. Wigler (Cold Spring Harbor, New York). The al-1 cell line is a secondary transformant obtained by transfection of NIH 3T3 cells with DNA isolated from T24 bladder carcinoma and it contains three or four copies of activated c-Ha-*ras* and *Alu* family sequences (13, 14). NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum at 37°C, whereas transformed NIH 3T3 cells were cultured in the same medium supplemented with 5% calf serum.

Preparation of DNA and Transfection Assay. High molecular weight DNA was extracted by the $NaDodSO_4$/proteinase K/phenol/chloroform method (15) from tissues or cultured cells. A surgical specimen of cervical cancer tissue was obtained from a 60-year-old patient undergoing hysterectomy for stage III cervical cancer without prior radiation therapy or chemotherapy. The cancer was diagnosed histologically as a moderately differentiated adenocarcinoma. DNA transfection into NIH 3T3 cells by the calcium phosphate coprecipitation technique was performed as described (16, 17). Transformed foci were scored 21 days after transfection. Clones of transformed cells were obtained from foci by cloning with the use of glass cylinders.

Tumorigenicity. NIH 3T3 cells or transformed cells were collected and suspended in serum-free Dulbecco's modified Eagle's medium. A total number of 1×10^6 cells in 0.2 ml of the medium (each) was injected subcutaneously into nude mice, and the mice were then examined every 2–3 days for tumors. In some experiments, the tumors were excised and their DNAs were examined for the presence of HPV type 16 and *Alu* sequences.

Southern Blot Hybridization. DNAs were digested with restriction endonucleases under the conditions suggested by the manufacturers. Samples of 20 μg were subjected t electrophoresis on a 0.8% agarose gel, transferred to nitrocellulose filters by the method of Southern (15, 18), and hybridized to a nick-translated ^{32}P-labeled *Bam*HI fragment of HPV 16 DNA (1) or human specific *Alu* family sequences purified from the plasmid BLUR8 (19, 20). *Hin*dIII-digested λ DNA was used as a size marker. A plasmid containing the entire DNA sequence of HPV type 16 with the size of 7.9 kilobase pairs (kbp) (1) was kindly provided by H. zur Hausen and L. Gissmann. The total HPV type 16 DNA sequence was cut out from the plasmid with *Bam*HI digestion, purified by electrophoresis and electroelution, and used as a probe. Subgenomic fragments used for probes were prepared by double digestion of cloned HPV type 16 DNA with *Bam*HI and *Kpn* I and by fractionation of the cleavage product in an agarose gel: 2.6-kbp *Bam*HI/*Kpn* I and 0.8-kbp *Kpn* I/*Bam*HI fragments (21). The specific activity of the probe was $>2 \times 10^8$ cpm/μg of DNA. Hybridization was performed with a HPV type 16 probe at a concentration of $> 2 \times 10^6$ cpm/ml in a solution of 50% formamide, 1.05 M sodium chloride/0.105 M sodium citrate, 0.2% $NaDodSO_4$, 5× concentrated Denhardt's solution (15), 10% dextran sulfate, and 100 μg of denatured salmon testis DNA per ml at 42°C for 20 hr. The filter was washed three times with 0.3 M sodium chloride/0.03 M sodium citrate containing 20 mM sodium phosphate (pH 7.0), 0.06% sodium pyrophosphate, and 0.05% $NaDodSO_4$ and once with 0.15 M sodium chloride/0.015 M sodium citrate containing the same components at 50°C. Then it was exposed to Kodak XRP-5 film with an intensifying screen at −70°C for 4–48 hr. Hybridization with the *Alu* family sequence was performed at a probe concentration of $>2 \times 10^6$ cpm/ml in 0.9 M sodium chloride/0.09 M sodium citrate, 0.1% $NaDodSO_4$, 5× concentrated Denhardt's solution, and 100 μg of denatured salmon testis DNA per ml at 69°C for 42 hr. The washing conditions were the same as for hybridization with the HPV type 16 DNA sequence, except that the temperature was 69°C.

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Abbreviations: HPV, human papillomavirus; kbp, kilobase pair(s); kb, kilobase(s).

Preparation and Analysis of RNA. Total RNA was prepared by the guanidinium isothiocyanate/CsCl method (22), and poly(A)$^+$ RNA was obtained by passing the total RNA through an oligo(dT)-cellulose column (23). Total RNA or poly(A)$^+$ RNA was electrophoresed through a 0.8% agarose gel containing 6.0% formaldehyde as described (15). Duplicate sets of RNA samples were subjected to electrophoresis. One set was used to examine the integrity of ribosomal RNA by staining with ethidium bromide. The other set was transferred to nitrocellulose filters (15, 24), and after fixation and prehybridization, hybridization was performed as described for Southern blot hybridization with the HPV type 16 DNA sequence.

RESULTS

The high molecular weight DNA used in the present studies was isolated from a cervical cancer that was diagnosed histologically as a moderately differentiated adenocarcinoma. Southern blot analysis of *Bam*HI-digested DNA of this sample showed a main band of 5.1 kbp with an additional band of 7.9 kbp, which hybridized to the HPV type 16 probe (Fig. 1*A*). From the intensity of the band, the copy number of the HPV type 16 DNA sequence was estimated to be ≈100 copies per haploid genome. Samples of 30 μg of this DNA were transfected into NIH 3T3 cells by the calcium phosphate coprecipitation technique. Three weeks later, a transformed focus composed of crisscrossed and piled up cells was detected (Fig. 2). The transformed cells were less refractile than those with DNA from al-1 cells containing activated c-Ha-*ras* from T24 bladder carcinoma. Because of the limited amount of cervical cancer tissue available, it was not possible

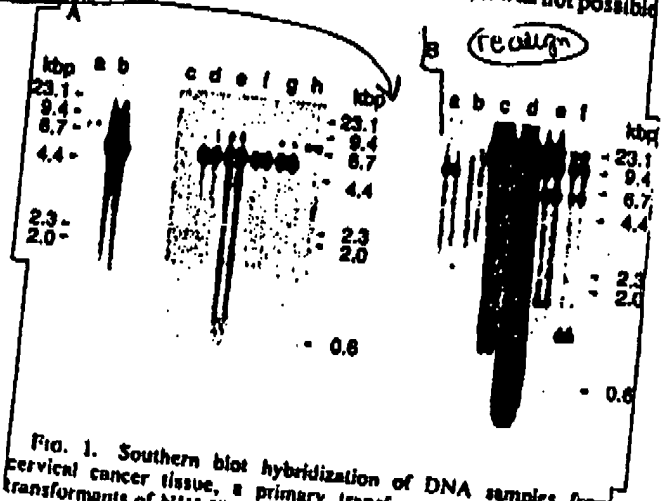

FIG. 1. Southern blot hybridization of DNA samples from cervical cancer tissue, a primary transformant, and secondary transformants of NIH 3T3 cells. DNAs were digested with *Bam*HI and 20 μg of each sample was subjected to electrophoresis on a 0.8% agarose gel, transferred to nitrocellulose filters, and hybridized to a nick-translated ^{32}P-labeled *Bam*HI fragment of HPV type 16 (*A*) or a human specific *Alu* sequence purified from the plasmid BLUR8 (*B*). (*A*) A sample of DNA containing 60 pg of HPV type 16 DNA and 20 μg of DNA from NIH 3T3 cells, corresponding to one copy of HPV type 16 sequence per human haploid genome, was included (lanes a and h). The DNAs analyzed were obtained from a human cervical cancer tissue (lane b), NIH 3T3 cells (lane c), a primary transformant of NIH 3T3 cells (lane d), a tumor formed in a nude mouse by injection of the primary transformant (lane e), secondary transformant 1 (lane f), and secondary transformant 2 (lane g). (*B*) DNAs were from NIH 3T3 cells (lane b), a primary transformant of NIH 3T3 cells (lane c), a tumor formed in a nude mouse by injection of the primary transformant (lane d), secondary transformant 1 (lane e), and secondary transformant 2 (lane f). DNA from al-1 cells containing three or four copies of *Alu* family sequences was used as a positive control for detection of *Alu* sequences (lane a).

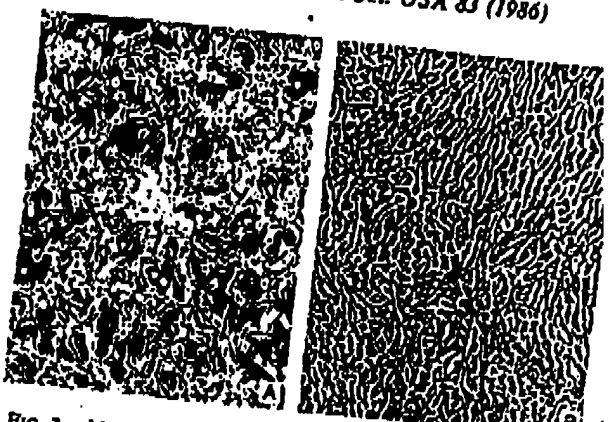

FIG. 2. Morphological appearance of a primary transformant (*A*) and NIH 3T3 cells (*B*).

to obtain further primary transformants by transfection. The transformed cells were picked up with a glass cylinder and grown, and their DNA was obtained. Southern blot hybridization showed that *Bam*HI digests of DNA from the transformant gave a major band of 5.1 kbp hybridized t the HPV type 16 DNA probe in addition to a faint band f 7.9 kbp (Fig. 1*A*). Furthermore, it was found that this DNA sample contained conspicuous bands that hybridized with human specific *Alu* family sequence (Fig. 1*B*). The transformant was highly tumorigenic to nude mice: tumors were formed in all mice tested 10 days after subcutaneous inoculation of 1 × 10^6 cells of the transformant. The DNAs from the tumors formed in nude mice also had *Alu* family sequences and HPV type 16 sequences (Fig. 1*B*). In contrast, the NIH 3T3 cells used f r transfection were not tumorigenic: no tumors developed within 8 weeks after inoculation of the same number of the cells.

The DNA obtained from the primary transformant induced transformed foci upon transfection into NIH 3T3 cells in a second cycle. Morphologically, these secondary transformants appeared to be the same as the primary transformant and they were highly tumorigenic to nude mice. Southern blot hybridization showed that *Bam*HI digests of DNAs of these secondary transformants contained a 5.1-kbp fragment that strongly hybridized to the HPV type 16 probe (Fig. 1*A*). Transformant 2 gave a 7.9-kbp fragment in addition t the 5.1-kbp fragment. DNAs from the primary transformant and the secondary transformants 1 and 2 were further analyzed by Southern blot hybridization after cleavage with *Pst* I, *Bam*HI/*Pst* I, and *Bam*HI/*Ava* II. Subgenomic fragments of HPV type 16 DNA, 2.6-kbp *Bam*HI/*Kpn* I, and 0.8-kbp *Bam*HI/*Kpn* I fragment were used as probes (Fig. 3). The results showed all of these three transformants contained the same subgenomic fragments of HPV type 16 DNA with the total sizes of at least 3.5 kbp, probably corresponding to 1.6-kbp, 0.2-kbp, and 1.7-kbp *Pst* I fragments of prototype HPV type 16 DNA sequence (21, 25). Furthermore, *Bam*HI digests of DNAs of these secondary transformants contained human specific *Alu* family sequences. Secondary transformant 1 contained 19-kbp, 9.4-kbp, 5.9-kbp, and 1.7-kbp fragments, and transformant 2 contained 19 kbp, 9.4 kbp, 5.9 kbp, 1.7 kbp, and 1.0 kbp (Fig. 1*B*). Integration of the HPV type 16 sequence into DNA of the primary and secondary transformants was further examined by Southern blot hybridization f DNAs from these transformants after cleavage with *Hin*dIII. HPV type 16 DNA had no restriction enzyme site for *Hin*dIII (21). With the 0.8-kbp *Kpn* I/*Bam*HI subgenomic fragment of HPV type 16 DNA as a probe, *Hin*dIII-digested DNAs from the primary and secondary transformants gave 12-kbp bands that were longer than the total length of this virus, whereas undigested DNA gave

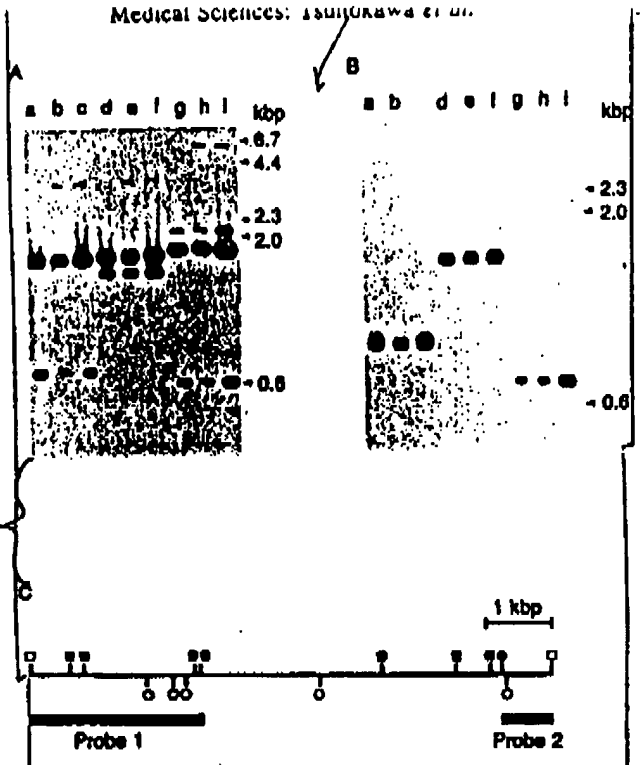

FIG. 3. Southern blot hybridization analysis of HPV type 16 DNA sequences in the transformants. DNAs from the secondary transformant 1 (lanes a, d, and g), the secondary transformant 2 (lanes b, e, and h), and the primary transformant (lanes c, f, and i) were digested with *Pst* I/*Bam*HI (lanes a–c), *Pst* I (lanes d–f), and *Bam*HI/*Ava* II (lanes g–i), and Southern blot hybridization analysis was performed with probe 1 (*A*) and probe 2 (*B*). Probe 1 and probe 2 were the 2.6-kbp *Bam*HI/*Kpn* I fragment and 0.8-kbp *Kpn* I/*Bam*HI fragment of cloned prototype HPV type 16 DNA, respectively (*C*). (*C*) Restriction map of cloned prototype HPV type 16 DNA (21), indicating cleavage sites for *Bam*HI (□), *Pst* I (■), *Ava* II (○), and *Kpn* I (●).

band in the position of high molecular weight DNA (data not shown). Considering these data, it is very unlikely that HPV type 16 sequences are present in the episomal form in the transformants.

RNA transfer blot analysis of poly(A)$^+$ RNA from the primary transformant showed that the transformed cells expressed HPV type 16 sequences with the size of 1.8 kilobases (kb). Additional faint bands corresponding to sizes of 6.8 kb, 4.9 kb, and 2.7 kb were also observed (Fig. 4).

DISCUSSION

Several recent reports have indicated the close association of HPV type 16 and type 18 with cervical cancers. HPV type 16 was reported to be associated with 62% of the cervical cancers examined from the Federal Republic of Germany, 35% of those from Kenya and Brazil (1, 3), and 45% of those from England (6). Moreover, 40–60% of cervical cancers in Japan were associated with HPV type 16 or type 18 (11, 26). Several cultured cell lines established from cervical cancers contained HPV type 16 or type 18 DNA sequences (7), including those established from Japanese patients (26). Furthermore, HPV type 16 or type 18 DNA sequences were found to be transcribed in these tissues or cells.

The present experiments showed the presence of transforming sequences in the DNA sample from the cervical cancer that could be transmitted through a second cycle of transfection to NIH 3T3 cells. The transforming sequences included human specific *Alu* family sequences and HPV type 16 DNA sequences, and the latter were expressed as

FIG. 4. Transcription of HPV type 16 genome in the transformant. Five micrograms of poly(A)$^+$ RNA was purified from a primary transformant (lane a) and NIH 3T3 cells (lane b), subjected to electrophoresis, transferred to a nitrocellulose filter, and hybridized with a nick-translated ^{32}P-labeled *Bam*HI fragment of HPV type 16 DNA. The locations of 18S (1.9 kb) and 28S (4.8 kb) are indicated.

poly(A)$^+$ RNA in the primary transformant. HPV type 16 DNA sequences with human genomic DNA were integrated into the primary and secondary transformants of NIH 3T3 cells. These results provide evidence showing that HPV type 16 sequences integrated into total genomic DNA from the cervical cancer had transforming activity when transfected into NIH 3T3 cells.

There are reports that bovine papillomavirus type 1 or its cloned full-length DNA transformed rodent cells *in vitro* and that the viral DNA remained as stable extrachromosomal plasmids in the transformed cells (27, 28). These reports suggested that parts of the bovine papillomavirus type 1 itself were responsible for the transforming activity. Despite of the potential importance of HPV type 16 DNA in development of cervical cancer, there were controversial reports on the transforming activity of HPV type 16 DNA. It was shown that HPV type 16 DNA sequence cloned in the pSV2$_{neo}$ failed to induce malignant transformation of NIH 3T3 cells (29). However, it was reported recently that a recombinant plasmid containing a head-to-tail HPV type 16 dimer at the *Bam*HI site of pSV2$_{neo}$ could induce malignant transformation when transfected into NIH 3T3 cells (12), indicating the HPV type 16 genome itself was responsible for the transforming activity. It is possible that linearizing HPV type 16 DNA at the *Bam*HI site might lead to disruption of its transforming activity. The possibility of insertional mutation-type activation of a cellular oncogene (30) by HPV type 16 DNA sequence cannot be ruled out at present for the acquisition of the transforming activity of the cervical cancer DNA reported here.

Studies are necessary to determine what part of HPV type 16 sequences is required for transformation of NIH 3T3 cells. The structure and the role of the human genome containing *Alu* family sequences associated with HPV type 16 DNA sequences should also be clarified.

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1. Dürst, M., Gissmann, L., Ikenberg, H. & zur Hausen, H. (1983) *Proc. Natl. Acad. Sci. USA* 80, 3812–3815.
2. Baird, P. J. (1983) *Lancet* II, 17–18.
3. Boshart, M., Gissmann, L., Ikenberg, H., Kleinheinz, A., Scheurlen, W. & zur Hausen, H. (1984) *EMBO J.* 3, 1151–1157.
4. Fukushima, M., Okagaki, T., Twiggs, L. B., Clark, B. A., Zachow, K. R., Ostrow, R. S. & Faras, A. J. (1985) *Cancer Res.* 45, 3252–3255.
5. Schwarz, E., Freese, U. K., Gissmann, L., Mayer, W., Roggenbuck, B., Stremlau, A. & zur Hausen, H. (1985) *Nature (London)* 314, 111–114.
6. Scholl, S. M., Kingsley Pillers, E. M., Robinson, R. E. & Farrell, P. J. (1985) *Int. J. Cancer* 35, 215–218.
7. Yee, C., Krishnan-Hewlett, I., Baker, C. C., Schlegel, R. & Howley, P. (1985) *Am. J. Pathol.* 119, 361–366.
8. Syrjänen, K., de Villiers, E.-M., Saarikosky, S., Castren, O., Väyrynen, M., Mäntyjärvi, R. & Parkkinen, S. (1985) *Lancet* I, 510–511.
9. Crum, C. P., Mitao, M., Levine, R. U. & Silverstein, S. (1985) *J. Virol.* 54, 675–681.
10. Schneider, A., Kraus, H., Schuhmann, R. & Gissmann, L. (1985) *Int. J. Cancer* 35, 443–448.
11. Yoshikawa, H., Matsukura, T., Yamamoto, E., Kawana, T., Mizuno, M. & Yoshiike, K. (1985) *Jpn. J. Cancer Res. (Gann)* 76, 667–671.
12. Yasumoto, S., Burkhardt, A., Doniger, J. & Dipaolo, J. A. (1985) *Proc. Am. Assoc. Cancer Res.* 26 (abstr.).
13. Santos, E., Tronick, S. R., Aaronson, S. A., Pulciani, S. & Barbacid, M. (1982) *Nature (London)* 298, 343–347.
14. Fasano, O., Birnbaum, D., Edlund, L., Fogh, J. & Wigler, M. (1984) *Mol. Cell. Biol.* 4, 1695–1705.
15. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
16. Wigler, M., Sweet, R., Sim, G. K., Wold, B., Pellicer, A., Lacy, E., Maniatis, T., Silverstein, S. & Axel, R. (1979) *Cell* 16, 777–785.
17. Perucho, M., Goldfarb, M., Shimizu, K., Lama, C., Fogh, J. & Wigler, M. (1981) *Cell* 27, 467–476.
18. Southern, E. M. (1975) *J. Mol. Biol.* 98, 503–517.
19. Houck, C. M., Rinehart, F. P. & Schmid, C. W. (1979) *J. Mol. Biol.* 132, 289–306.
20. Rubin, C. M., Houck, C. M., Deininger, P. L., Friedmann, T. & Schmid, C. W. (1980) *Nature (London)* 284, 372–374.
21. Dürst, M., Kleinheinz, A., Hotz, M. & Gissmann, L. (1985) *J. Gen. Virol.* 66, 1515–1522.
22. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979) *Biochemistry* 18, 5294–5299.
23. Aviv, H. & Leder, P. (1972) *Proc. Natl. Acad. Sci. USA* 69, 1408–1412.
24. Thomas, P. S. (1980) *Proc. Natl. Acad. Sci. USA* 77, 5201–5205.
25. Seedorf, K., Krämmer, G., Dürst, M., Suhai, S. & Röwekamp, W. G. (1985) *Virology* 145, 181–185.
26. Tsunokawa, Y., Takebe, N., Nozawa, S., Kasamatsu, T., Gissmann, L., zur Hausen, H., Terada, M. & Sugimura, T., *Int. J. Cancer*, in press.
27. Sarver, N., Rabson, M. S., Yang, Y.-C., Byrne, J. C. & Howley, P. M. (1985) *J. Virol.* 52, 377–385.
28. Yang, Y.-C., Okayama, H. & Howley, P. M. (1985) *Proc. Natl. Acad. Sci. USA* 82, 1030–1034.
29. Chesters, P. M. & McCance, D. J. (1985) *J. Gen. Virol.* 66, 615–620.
30. Hayward, W. S., Neel, B. G. & Astrin, S. M. (1981) *Nature (London)* 290, 475–480.

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The *trans*-inhibitory Rep78 protein of adeno-associated virus binds to TAR region DNA of the human immunodeficiency virus type 1 long terminal repeat

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Abstract The large *rep* gene products, Rep78 and Rep68, of adeno-associated virus (AAV) are pleiotropic effector proteins which are required for AAV DNA replication and the *trans*-regulation of AAV gene expression. Apart from these essential functions prerequisite for the life cycle of AAV, these *rep* products are able to inhibit the replication and gene expression of human immunodeficiency virus type 1 (HIV-1) and a number of DNA viruses. Here, it is demonstrated that Rep78, as a chimeric with the maltose binding protein, directly binds the full-length HIV-1 long terminal repeat (LTR), and to a subset of these sequences containing the *trans*-activation response (TAR) sequence as DNA. These interactions, an effector protein physically binding a target promoter, suggest a direct mechanism of action for Rep78 inhibition. Furthermore, competitive binding studies between the TAR region and the full-length HIV-LTR, strongly suggested that another site(s) within the LTR was also bound by Rep78. Finally, as Rep78 binding is also believed to be affected by secondary structure within the DNA, it was found that Rep78 preferentially binds with HIV-LTR sequences with promoted secondary structure generated by heat denaturation and rapid cooling.

Key words: Human immunodeficiency virus; Adeno-associated virus; Rep78; *trans*-Activation response element

1. Introduction

AAV's life cycle as a human helper-dependent parvovirus requires that it interacts with other viruses, and it might generally be thought of as a parasite of its helpers, adeno- and herpes simplex virus [1,2]. AAV has been found to inhibit a variety of viruses, including, adenovirus [3,4], herpes simplex virus [5,6], papillomavirus [7–9], and HIV-1 [10–14]. The Rep78 and Rep68 proteins, encoded by the AAV *rep* gene, have been found to be responsible for these inhibitions [7–9]. Furthermore, Rep78/68 regulate the expression of cellular genes, including c-H-*ras* [15–17], c-*fos* [18,19], and c-*myc* [18,19]. These finding are consistent with the Rep78/68's function as regulators of AAV's own gene expresson.

Previous studies of AAV's Rep78/68 inhibition of HIV-1 have concluded that both HIV-1 gene expression and replication are inhibited [10–14]. During plasmid co-transfection and micro-injection experiments the wild type AAV genome will inhibit HIV-1 replication from 90 to 99%, depending upon the particular study. However, Rep78/68 inhibition of HIV-1 gene expression and replication is even stronger when Rep78/68 are expressed from the HIV-1 long terminal repeat itself (LTR) [12]. Although AAV's inhibition of HIV-1 is well documented, the mechanism by which Rep78/68 inhibit HIV-1 has not been determined. Rep78/68 are highly multifunctional proteins with activities which include covalent and non-covalent binding to DNA, ATP-dependent helicase activity, site-specific endonuclease activity, and transcriptional *trans*-activation and *trans*-repression [20–25]. It is likely that Rep78/68's function as transcriptional regulators are involved in the mechanism of inhibition of HIV-1.

Thus far, it is believed that the short DNA motif, GCTC, is involved in the recognition of DNA by Rep78/68. This sequence is present as a trimeric concatemer in the terminal repeats (TR) of AAV and near the site of AAV preferential integration on human chromosome 19 [22,23,26–28]. The number of GCTC motifs, in close proximity, appear to partially determine the affinity of Rep78/68 protein binding [29]. However, secondary structure within the DNA also plays a role in DNA recognition by Rep78/68. It has been noticed by others [11] that the *cis* TAR sequence of HIV-1, to which the HIV-1 encoded *tat trans*-activator binds as RNA, contains multiple GCTC motifs [30,31]. This laboratory has been studying Rep78 functions utilizing a maltose binding protein-Rep78 chimeric (MBP-Rep78), produced in bacteria, which has all the known biochemical functions of wild type Rep78 from eukaryotes [32]. In this study we demonstrate that MBP-Rep78 is able to bind to the TAR region of HIV-1 as DNA.

2. Materials and methods

2.1. Preparation of DNA substrates and MBP-Rep78 protein

The AAV TR and murine osteosarcoma virus (MSV)-LTR substrates (the positive and negative controls for Rep78 binding, respectively) have been described previously [32], and their sequences are shown in Fig. 1. The full-length HIV-LTR substrate (nt −454 to +42) was generated by PCR amplification with Taq DNA polymerase, using linearized pBennCAT plasmid [33] (*Bam*HI digested) as a template and the primers i and ii, shown in Fig. 1. This substrate is referred to as HIV-LTR (PCR) and was purified by Qiaquick spin column (Qiagen Inc.). Complementary strands of synthetic DNA oligonucleotides (oligo)(Operon) ii and iii of Fig. 1 were annealed to generate the TAR^A substrate (nt +23 to +42). A third HIV-1 DNA substrate was generated by PCR amplification utilizing primers iii and iv, and referred to as TAR^B (+23 to +86). When appropriate, the substrates were labeled utilizing polynucleotide kinase and [γ-^{32}P]ATP. The production and purification of the chimeric MBP-Rep78 protein used in the study has been described elsewhere [32].

2.2. PCR amplification

Amplification was performed in 50 μl reaction mixtures containing

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50 mM KCl, 10 mM Tris-HCl pH 8.8, at 25°C, 2 mM $MgCl_2$, 150 mM each of dCTP, dATP, dGTP and dTTP, 100 pmol of both primers (shown in Fig. 1), 1 ng of template (*Bam*H I linearized pBennCAT), and 2.5 units of Taq DNA polymerase (Promega, Madison, WI) overlaid with mineral oil. The first denaturing step was performed at 94°C for 2 min. A total of 30 cycles were performed with a denaturing temperature at 92°C for 40 s, annealing at 60°C for 40 s and extension for 1.5 min at 75°C. Final extension was performed for 5 min. The PCR product was purified by Qaiquick spin column (Qiagen Inc.).

2.3. Electrophoretic mobility shift assay (EMSA)

EMSA assays were conducted as described previously with minor modifications [34]. Approximately 1 ng of 5′ end-labeled DNA substrate was incubated with increasing amounts of MBP-Rep78 for 10 min at room temperature in binding buffer [(25 mM HEPES KOH pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreiotol, 2% glycerol, 25 μg bovine serum albumin, 50 mM NaCl, 0.01% NP40 and 0.5 μg poly (dI–dC)]. Samples were electrophoresed in a 4% polyacrylamide gel (40:1 acrylamide and bis-acrylamide weight ratio) with 5% glycerol in 0.5 × TBE buffer at 100 V for about 3 h. Gels were dried and autoradiographed at −70°C. Addition of increasing amounts of MBP-Rep78 is indicated in the figure legend. To promote DNA secondary structure formation in the HIV-LTR (PCR) substrate was boiled for 2 min and chilled immediately on ice as described by others [35]. Experiments involving competition between radiolabeled substrate and unlabeled substrate were carried out as indicated in the specific figure.

3. Results

3.1. MBP-Rep78 binds to the full-length HIV-LTR

With the knowledge that Rep78 recognizes GCTC motifs (or its complement, GAGC) it was observed that the HIV-LTR contained 8 of these motifs. Fig. 1 shows the sequences of the HIV-LTR, the GCTC motifs, and the HIV-1 derived synthetic DNA oligonucleotides used in this study. T determine whether Rep78 bound anywhere within the full-length HIV-LTR, the sequences from nt −454 to nt +42 were amplified by polymerase chain reaction (PCR) and tested in an electrophoretic mobility shift assay (EMSA) for binding with a Rep78/maltose binding protein chimeric, MBP-Rep78. This chimeric MBP-Rep78 protein has identical biochemical activities to that of the fully wild type Rep78 in all assays so far attempted (DNA binding, helicase, endonuclease activity, etc.) [25,26]. As shown in Fig. 2 the MBP-Rep78 protein was able to bind the HIV-LTR (PCR) as indicated by the appearance of a new high shifted band which was dependent of the amount of MBP-Rep78 added to the reaction. This interaction of the effector molecule with the target promoter DNA suggests a direct mechanism of action for AAV Rep78 inhibition of HIV replication and gene expression. The MBP protein did not bind this or any other DNA (data not shown).

3.2. MBP-Rep78 binds the HIV TAR DNA sequences

One region of the HIV-LTR was of particular interest as it contained three GCTC motifs (nt +25 to +38, motifs 1–3, Fig. 1B) in close proximity. This area corresponds to the *cis* TAR region, which is where the HIV-1 encoded *tat trans*-regulatory protein binds as an mRNA sequence, resulting in > 100-fold

AAV TR: (positive control)

5′...G_{145}AACCCCTAGTGATGGAGTTGGCCACTCCCTCTCTGCGC***GCTC***^{3}***GCTC***^{2}***GCTC***^{1}ACTGAGGC$_{85}$

MSV-LTR: (negative control)

5′...C_{58}TGTACCCGCGCTTATT***GCTC***^{1}TATAAAAAGGGTAAGAACCCCACACTCGGCG$_{+1}$

HIV-LTR:

5′ T$_{-454}$GGAAGGGCTAATTCACTCCC$_{-434}$AACGAAGACAAGATATCCTTGATCTGTGGATCTACCACA
CACAAGGCTACTTCCCTGATTAGCAGAACTACACACCAGGGCCAGGGGTCAGATATCCACTGACCT
TTGGATGGTGCTACAAGCTAGTACCAGTT***GAGC***^{6}CAGATAAGGTAGAAGAGGCCAATAAAGGAGAG
AA CACCAGCTTGTTACACCCTGT***GAGC***^{5}CTGCATGGGATGGATGACCCGGAGAGAGAAGTGTTAGA
GTGGAGGTTTGACAGCCGCCTAGCATTTCATCACGTGGCCCGA***GAGC***^{4}TGCATCGGGAGTACTTCAA
GAACTGCTGATATC***GAGC***^{3}TTGCTACAAGGGACTTTCCGCTGGGGACTTTCCAGGGAGGCGTGGCCT
GGGCGGGACTGGGGAGTGGC***GAGC***^{2}CCTCAGATCCTGCATATAAGCAGCTGCTTTTTGCCTGTACTG
⌜ G$_{+1}$GTCTCTCTGGTTAGACCAGATC$_{+23}$T***GAGC***^{3}CTGG***GAGC***^{2}***TC***^{1}TCTG$_{+42}$GCTAGCTAGGGAACC
CACTGC$_{+63}$TTAAGCCTCAATAAAGCTGCCTT$_{+88}$ 3′

HIV-1 oligonucleotides:

i 5′ TGGAAGGGCTAATTCACTCCC
ii 5′ CAGAGAGCTCCCAGGCTCAG
iii 5′ CTGAGCCTGGGAGCTCTCTG
iv 5′ AAGGCAGCTTTATTGAGGCTTAAG

Fig. 1. Sequences of the DNA substrates and synthetic oligonucleotides used in this study. At the top of the figure are sequences from the AAV TR and murine osteosarcomavirus (MSV) LTR. The underlined bases represent substrate sequences. The motifs GCTC and GAGC are in italics, bolded and identified by superscript numbers. The AAV TR and MSV-LTR substrates were used as the positive and negative controls, respectively [29]. Also shown are the sequences of the HIV-1 LTR. The underlined bases in the HIV-LTR represent substrate sequences and/or PCR primers used to generate substrates. The nucleotide positions relative to the transcription initiation site (bent arrow) of the HIV-LTR promoter are denoted by subscript numbers. The oligonucleotides i and ii were used to PCR generate the HIV-LTR (PCR) substrate (nt −454 to +42). The oligonucleotides ii and iii were used together (complementary) as the TAR^A oligonucleotide substrate (nt +23 to +42), which encompasses the TAR sequences. The oligonucleotides iii and iv were used to PCR generate the TAR^B substrate (Nt +23 to +86). The plasmid pBennCAT was the PCR template.

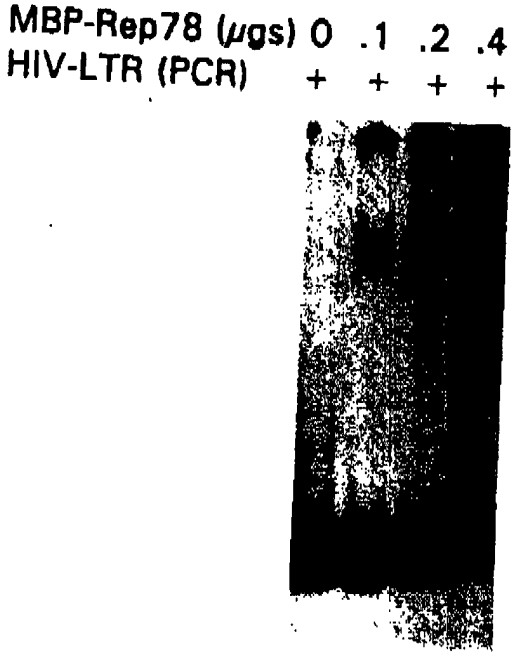


Fig. 2. Dosage dependent complex formation between HIV-LTR DNA sequences and MBP-Rep78 protein. Note that increasing amounts of the band shifted MBP-Rep78/HIV-LTR (PCR) complex appear with increasing amounts of MBP-Rep78. The HIV-LTR substrate (nt −454 to +42) was generated by PCR amplification with Taq DNA polymerase, using linearized pBennCAT plasmid (*Bam*HI digested) as a template and the primers i and ii, shown in Fig. 1. MBP-Rep78 protein production and purification has been described previously [32]. EMSA assays were conducted as described in section 2. Briefly, 1 ng of ^{32}P-5′-labeled HIV-LTR (PCR) was incubated with increasing amounts of MBP-Rep78 for 10 min at room temperature in binding buffer. Samples were electrophoresed in a 4% polyacrylamide gel, dried and autoradiographed. Addition of increasing amounts of MBP-Rep78 indicated above the panel.

higher expression levels [30,31]. This laboratory has previously demonstrated that a region of the c-H-*ras* promoter, with a similar three motif architecture, was able to bind an MBP-Rep protein chimeric [2]. To observe if sequences from the TAR region of the HIV-LTR were able to bind MBP-Rep78, a synthetic duplex DNA oligonucleotide substrate (Fig. 1A, ii and iii, and C from nt +23 to +42, referred to as TAR^A, was used as a substrate for MBP-Rep78 recognition in an EMSA (Fig. 3). The results show that MBP-Rep78 does bind the TAR^A sequences, as indicated by a shifted band. However, the affinity of MBP-Rep78 for TAR^A was at an intermediate level compared to the AAV TR (positive control, the natural substrate for Rep78, with 3 GCTC motifs in a concatemeric arrangement [29]) and a selected sequence from the MSV-LTR (negative control, one GCTC motif [29], also referred to as 'MSV').

In addition to the three GCTC motifs present in the AAV TR's stem, the secondary structure of the TRs, with multiple hairpin loops, are believed to play a role in the recognition and binding affinity of Rep78. Furthermore, this laboratory has demonstrated binding to a single duplex DNA GCTC motif within the context of a DNA hairpin [28]. The DNA sequence of the HIV-1 TAR region also shows the potential for significant secondary structure in the region from nt −1 to +82. To investigate whether Rep78 bound to this larger TAR sequence, a substrate larger than TAR^A was generated by PCR amplification and called TAR^B (from nt +23 to +86) utilizing oligonucleotides iii and iv (Fig. 1A). TAR^B did not contain any additional GCTC motifs over TAR^A. When assayed in the EMSA, the TAR^B sequence also was recognized by MBP-Rep78, and at a slightly elevated level compared to TAR^A (Fig. 3).

3.3. Competition studies between the full-length HIV-LTR (PCR) and the TAR^A oligonucleotide substrate suggest that MBP-Rep78 is binding to additional sites within the HIV-LTR

As five other GCTC motifs are present within the HIV-LTR, in addition to those in the TAR region, the possibility that the TAR^A sequences were the major binding site for Rep78 within the HIV-LTR was investigated by EMSA competition studies. The small TAR^A duplex substrate was used as a unlabeled competitor against Rep78 binding with the full-length ^{32}P-labeled HIV-LTR (PCR generated). The AAV TR and MSV-LTR synthetic duplex DNAs were also used as control competitors (positive and negative controls, respectively). As expected the AAV TR was an effective competitor, while the MSV-LTR was ineffective (Fig. 4A). TAR^A was also a very weak competitor. TAR^A was next compared directly with the full-length HIV-LTR (PCR generated) for effectiveness as a competitior (Fig. 4B). As expected the HIV-LTR competed effectively against itself, while TAR^A did not. These data are consistent with the hypothesis that Rep78 is also binding to other regions of the HIV-LTR, possibly other GCTC motifs, and with higher infinity than to the TAR region.

3.4. MBP-Rep78 preferentially binds to HIV-LTR substrates with promoted secondary structure

As mentioned previously, it has been reported that Rep78/68 protein binding to DNA subtrates is accentuated if those substrates have significant secondary structure, as with the AAV TR which will form a cruciform structure [26]. To determine

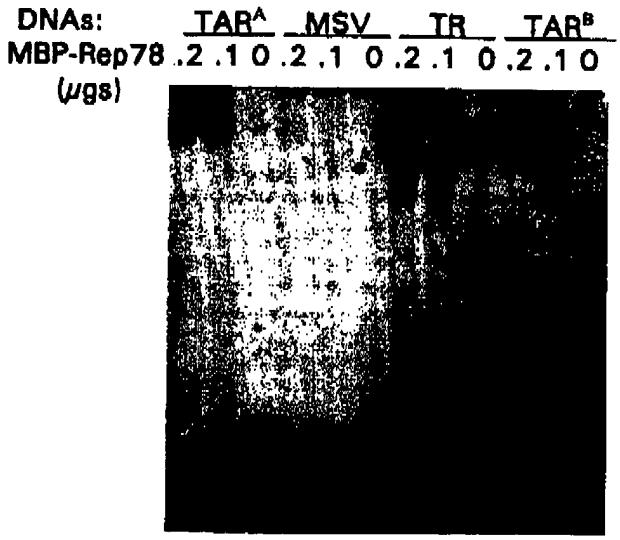
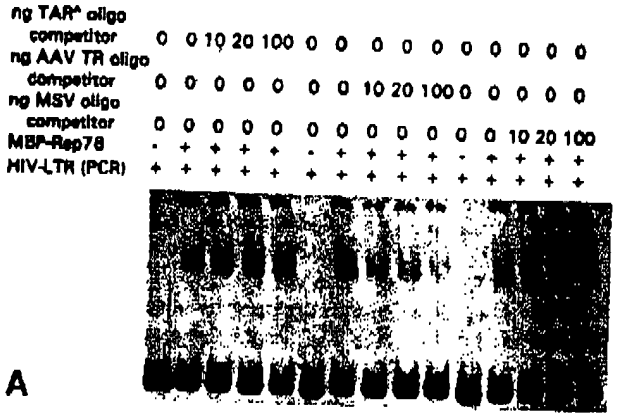


Fig. 3. Analysis of MBP-Rep78 binding to HIV TAR DNA. Note that a band shifted protein-DNA complex appears in both the TAR^A and TAR^B lanes indicating MBP-Rep78 binding to TAR. Approximately 1 ng of the indicated ^{32}P-5′-labeled substrate was incubated in the reaction. The TR and MSV-LTR are the positive and negative controls, respectively. EMSA assay was performed as mentioned in Fig. 2 with a 5% polyacrylamide gel.



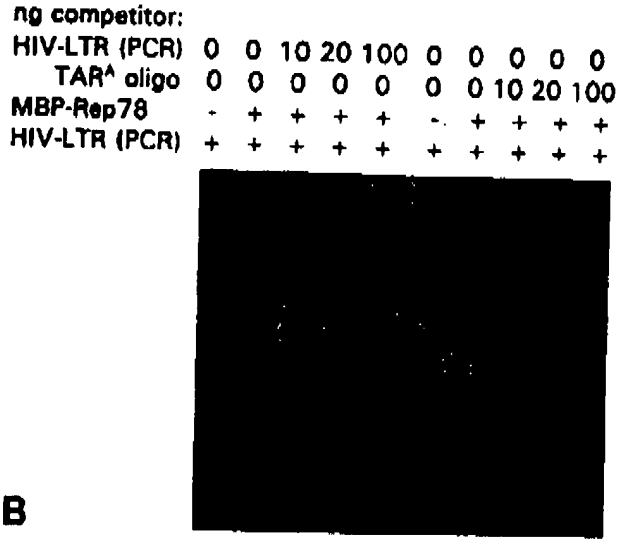


Fig. 4. Competition studies between the HIV-LTR and other DNA substrates for Rep78 binding. (A) Competition studies between ^{32}P 5'-labeled and unlabeled HIV-LTR (PCR) and with unlabeled TAR^{A}, for MBP-Rep78 binding activity. Note that the HIV-LTR (PCR) is a better competitor than TAR^{A}. Increasing amounts of unlabeled oligonucleotide competitors were added in the EMSA assay as indicated with equal amounts of MBP-Rep78 protein (500 ng). Approximately 1 ng of ^{32}P-5'-labeled HIV-LTR (PCR) substrate was incubated in every reaction. (B) Competition studies between ^{32}P-5'-labeled HIV-LTR (PCR) and unlabeled AAV terminal repeat (TR) or the MSV-LTR oligonucleotides for MBP-Rep78 binding. Note that the AAV TR oligonucleotide is an effective competitor when compared to the MSV-LTR oligonucleotides or the TAR^{A} oligonucleotide. Increasing amounts of unlabeled oligonucleotide competitors were added in the EMSA assay as indicated with equal amounts of MBP-Rep78 protein (500 ng). Approximately 1 ng of ^{32}P-5' end-labeled HIV-LTR (PCR) substrate was incubated in every reaction.

if secondary structure had an affect on MBP-Rep78 recognition of the full-length HIV-LTR, the HIV-LTR (PCR) substrate was heat denatured and quick-chilled on ice to generate 'snap back' secondary hairpin structures [35]. This 'snap back' HIV-LTR substrate was compared to normal duplex HIV-LTR (PCR) for Rep78 binding in an EMSA (Fig. 5). As shown in Fig. 5, the normal duplex and 'snap back' HIV-LTR (PCR) substrates migrated at different positions in the gel so that it was possible to distinguish between them. In one set of reactions equal amounts of the 'snap back' substrate and normal duplex substrate were added together in the EMSA. Both the normal duplex and the 'snap back' HIV-LTR (PCR) substrates were able to bind MBP-Rep78. However, when the duplex and 'snap back' substrates were together in the same reaction tube the 'snap back' substrate was preferentially band shifted by MBP-Rep78 demonstrating a higher affinity for this substrate.

4. Discussion

HIV infection is a global problem with no effective treatment. The inhibitory properties of AAV (Rep78) on HIV-1 replication and gene expression have been well documented. however, the specific mechanism of action is unknown. Our results demonstrate that the Rep78 *trans*-effector protein physically binds to target promoter sequences. Although the functional implications of Rep78 binding to TAR DNA (not TAR RNA to which *tat* binds) of the HIV-LTR are still unknown, these results suggest a direct mechanism of action for Rep78 *trans*-regulation of HIV. Rittner et al. (1992) have previously predicted that the Rep78/68 proteins may bind to specific sequences of the HIV-LTR [11]. Our data supports their hypothesis. The sequences of the HIV TAR oligonucleotides (Fig. 1) which we have shown binding to MBP-Rep78 are nearly identical to those predicted sequences. Possible detailed mechanisms for Rep78 inhibition may include the following: (i) the steric hindrance by Rep78 of the binding of important positive *trans*-regulators (e.g. *tat*), (ii) the signalling of other, more direct, effectors of negative *trans*-regulation, (iii) the direct interaction and effect of Rep78 with the RNA polymerase II complex of proteins, or (iv) the physical disruption of the HIV-1 DNA sequences by the Rep78 endonuclease function.

The inability of TAR^{A} to effectively compete against the full-length HIV-LTR (PCR) suggests that Rep78 is binding to additional sites within the HIV-LTR and with higher affinity than Rep78 recognition of TAR. Preliminary experiments in this laboratory are consistent with Rep78 strongly binding to another site within the HIV-1 LTR, near TAR (Batchu and Hermonat, unpublished). Furthermore, this study demonstrates that the HIV-LTR, with promoted secondary structure generated by rapid denaturation/renaturation, is preferentially bound by Rep78. These finding are consistent with studies of Rep78/68 binding to the AAV TR and to sequences from the c-H-*ras* promoter [20–23,29]. To what extent these secondary stuctures actually form in vivo is unknown and predicting the extent and importance of the these structures would be highly spectulative. However, the 'snap back' form may have relevance to HIV-1 gene expression as some transcription factors are known to recognize interrupted palindromes which have the potential to generate secondary structures (e.g. the papillomavirus E2 *trans*-activator which recognizes the sequence ACCN$_6$GGT [36].

Continuing these studies we intend to uncover Rep78's mechanism of action for inhibiting HIV-1. Ultimately, it is hoped that AAV Rep78 may be used as a treatment for HIV infection. As a gene therapy vector, another interesting area of AAV research [37], AAV is able to transduce bone marrow progenitor cells [38]. By placing the Rep78 gene under the regulation of an HIV responsive promoter and then transducing hematopoietic progenitor cells, it may be possible to construct an HIV resistant immune system, by generating intracellular immunization.

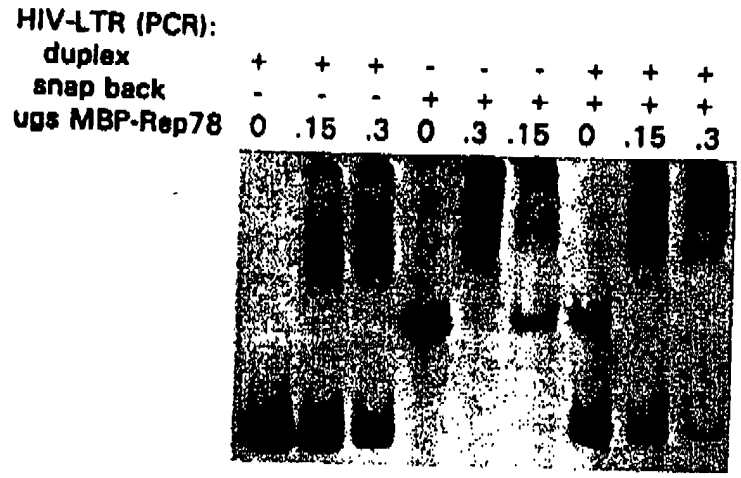


Fig. 5. Preferential binding of MBP-Rep78 with promoted secondary structure of HIV-LTR DNA compared to the same substrate without such promoted structures. Note that the HIV-LTR (PCR) with promoted secondary structure, the higher band compared to the normal duplex, disappears first with the addition of MBP-Rep78 protein. The HIV-LTR (PCR) was subjected to over 100°C for 2 min and quickly chilled on ice to form a 'snap back' secondary structure prior to the EMSA assay. Approximately 1 ng of labeled HIV-LTR (PCR) was used in the study. When normal duplex and promoted secondary structure HIV-LTR (PCR) were both added into one reaction, 0.5 ng of each substrate was used.

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References

[1] Buller, R.M., Janik, J.E., Subring, E.D. and Rose, J.A. (1981) J. Virol. 40, 241–247.
[2] Casto, B.C., Atchinson, R.W. and Hammon, W.McD. (1967) Virology 18, 52–60.
[3] Kirchstein, R.L., Smith, K.O. and Peters, E.A. (1968) Proc. Soc. Exp. Biol. Med. 128, 670–674.
[4] Casto, B.C., Atchinson, R.W. and Hammon, W.McD. (1967) Virology 18, 52–60.
[5] Cukor, G., Blacklow, N.R., Kibrick, S. and Swan, I.C. J. Natl. Can. Inst. 55, 957–959.
[6] Blacklow, N.R., Cukor, G., Kibrick, S. and Quinnan, G. (1978) in: Replication of Mammalian Parvovirues (D.C. Ward and P. Tattersal, Eds.) pp. 87–97.
[7] Hermonat, P.L. (1989) Virology 172, 253–261.
[8] Hermonat, P.L. (1992) Virology 189, 329–333.
[9] Hermonat, P.L. (1994) Cancer Res. 54, 2278–2281
[10] Oelze, I., Rittner, K. and Sczakiel, G. (1994) J. Virol. 68, 1229–1233.
[11] Rittner, K., Heilbronn, R., Kleinschmidt, J.A. and Sczakiel, G. (1992) J. Gen. Virol. 73, 2977–2981.
[12] Antoni, B.A., Rabson, A.B., Miller, I.L., Trempe, J.P. Chejanovsky, N. and Carter, B.J. (1991) J. Virol. 64, 396–404.
[13] Rittner, K., Heilbronn, R., Kleinschmidt, J.A., Oelze, I. and Sczakiel, G. (1991) Biochem. Soc. Transact. 19, 438S.
[14] Mendelson, E., Grossman, F., Mileguir, F., Rechavi, G. and Carter, B.J. (1992) Virology 187, 453–463.
[15] Katz, E. and Carter, B.J. (1986) Cancer Res. 46, 3023–3026.
[16] Hermonat, P.L. (1991) Can. Res. 151, 3373–3377.
[17] Khleif, S.N., Myers, T., Carter, B.J. and Trempe, J.P. (1991) Virology 181, 738–741.
[18] Hermonat, P.L. (1994) Cancer Lett. 81, 129–136.
[19] Klein-Bauernschmitt, P., zur Hausen, H. and Schlehofer, J.R. (1992) J. Virol. 66, 4191–4200.
[20] Im, D.S. and Muzyczka, N. (1989) Cell 61, 447–457.
[21] Im, D.S. and Muzyczka, N. (1992) J. Virol. 66, 1119–1128.
[22] Snyder, R.O., Im, D.-S., Ni, T., Xiao, X., Samulski, R.J., Muzyczka, N. (1993) J. Virol. 67, 6096–6104.
[23] Snyder, R.O., Im, D.S., Muzyczka, N. (1990) J. Virol. 64, 6204–6213.
[24] Labow, M.A., Hermonat, P.L. and Berns, K.I. (1986) J. Virol. 60, 251–258.
[25] Tratschin, J.D., Tal, J. and Carter, B.J. (1986) Mol. Cell Biol. 6, 2884–2894.
[26] Weitzman, M.A., Kyostio, S.R.M., Kotin, R.M. and Owens, R.A. (1994) Proc. Natl. Acad. Sci. USA 91, 5808–5812.
[27] Samulski, R.J., Zhu, X., Brook, J.D., Housman, D.E., Epstein, N. and Hunter, L.A. (1991) EMBO. J. 10, 3941–3950.31.
[28] Kotin, R.M., Linden, R.M. and Berns, K.I. (1992) EMBO. J. 11, 5071–5078.
[29] Batchu, R.B., Kotin, R.M. and Hermonat, P.L. (1994) Can. Lett. 86, 23–31.
[30] Hauber, J. and Cullen, B. (1988) J. Virol. 62, 673–679.
[31] Rosen, C.A., Sodroski, J.G. and Haseltine, W.A. (1988) Cell 41, 813–823.
[32] Batchu, R.B., Miles, D.A., Rechtin, T.M., Drake, R.R. and Hermonat, P.L. (1995) Biochem. Biophy. Res. Commun. 208, 714–720
[33] Gendelman, H.E. et al. (1986) Proc. Natl. Acad. Sci. USA 89, 9759–9763.
[34] Berkhout, B., Silverman, R.H. and Jeang, K.T. (1989) Cell 59, 273–282.
[35] Willwand, K. and Hirt, B. (1991) J. Virol. 65, 4269–4235.
[36] Spalholz, B.A., Yang, Y.C. and Howley, P.M. (1985) Cell 42, 183–191.
[37] Hermonat, P.L. and Muzyczka, N. (1984) Proc. Natl. Acad. Sci. USA 81, 6466–6470.
[38] La Face, D., Hermonat, P., Wakeland, E. and Peck, A (1988) Virology 162, 483–486.

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The regulatory *rep* protein of adeno-associated virus binds to sequences within the c-H-*ras* promoter

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Abstract

The large *rep* gene products (*rep*68 and *rep*78) of adeno-associated virus (AAV) are pleiotropic effector proteins which not only play a critical role in AAV DNA replication and in the *trans*-regulation of AAV promotor elements, but are also known for their onco-suppressive functions. We have previously demonstrated that the large AAV *rep* protein will strongly inhibit expression from the c-H-*ras* promoter, but not the murine osteosarcoma virus long terminal repeat (MSV-LTR) promoter. To investigate the possibility that *rep* may physically bind to these promoter sequences, specifically to GCTC motifs, we conducted electrophoretic mobility shift assays (EMSA) with a maltose binding protein-*rep* chimeric protein, MBP-*rep*68Δ, and synthetic double stranded DNA substrates of sequences selected from the c-H-*ras* and MSV-LTR promoters, as well as with the AAV TR. We find that MPB-*rep*68Δ bound the AAV TR DNA sequence (three motifs) most strongly, followed by the selected c-H-*ras* DNA sequence (two non-interfering motifs), and most poorly to the MSV-LTR DNA (one motif). These data are consistent with our previous study and suggest a direct mechanism of action for AAV *rep* inhibition of the c-H-*ras* promoter. Furthermore, the results suggest that the number of GCTC motifs, when closely associated, affect the affinity of *rep* binding. Finally, we find that MBP-*rep*68Δ also binds to the c-H-*ras* oligomer substrates which have secondary hairpin structures.

Keywords: Adeno-associated virus; *rep*; Transcription factor; c-H-*ras*; Anti-oncogene; DNA binding

1. Introduction

Adeno-associated virus (AAV) is a non-pathogenic, helper dependent, human parvovirus, which has anti-tumor and anti-proliferatory properties [7,8,36]. AAV has been shown to suppress tumor growth in animals [5,10,34,37], to decrease focus formation (transformation) of contact inhibited cells by viral or cellular oncogenes and to inhibit viral or carcinogen induced gene amplification [13–17,22,25,27–29]. Furthermore, sero-epidemiologic studies indicate that AAV infection is

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linked to lower cervical cancer rates [12,33]. The left half of the AAV genome comprises the *rep* gene open reading frame which encodes 4 overlapping proteins, of which the larger pair of related *rep* proteins, *rep*68/*rep*78, are responsible for AAV's anti-tumor/anti-cancer activities [15,27]. The large *rep* proteins are also responsible for the inhibition of herpes simplex virus [2,10], adenovirus [8,37], papillomavirus [14,15,17], and human immunodeficiency virus replication [1,38]. However, the large *rep* proteins are required for AAV DNA replication and for rescue from the chromosomally integrated provirus [18,19]. Because of the anti-tumor properties of the large *rep* gene products, they have been described as viral anti-oncogenes [15,24].

The large AAV *rep* proteins, *rep*68 and *rep*78, are highly multifunctional with biochemical activities which include ATP-dependent, site-specific strand-specific endonuclease activity. These proteins nick within the AAV terminal repeat (TR) terminal resolution site (*trs*), with concomitant covalent attachment to the 5′ end of the nick by a tyrosine–phosphate linkage [22,40,41]. This *trs* activity is required for AAV DNA replication and AAV *rep* mutants in this activity fail to generate either duplex replicating form or progeny single strand AAV DNA [35]. In addition, the purified *rep* proteins have an associated ATP-dependent DNA helicase activity [9,21,22]. Finally, the *rep* proteins are known to positively and negatively regulate AAV gene expression [3,4,30,42,43].

One of the questions which remained unclear was how the *rep* recognized its substrate(s). Earlier work from different laboratories suggested that the ability of the terminal repeat to form the characteristic T-shaped cruciform structure was essential for the *rep* binding [6,20,32,40]. A recent report indicates that the requirements for binding activity are not limited to recognition of the secondary structure, but sequences at the site of binding are necessary for specific recognition of the *rep* proteins [9]. DNAase I footprint experiments indicated the presence of GCTC triplet concatamer motif at the site of *rep* binding to terminal repeats [9]. Also, the similar sequences were observed at the AAV integration site at the human chromosome 19 [26,27,39,45].

Previous work by others and ourselves indicate that the large AAV *rep* protein(s) down-regulate the c-H-*ras* promoter [16,23,24], but have little effect on the murine osteosarcoma virus long terminal repeat promoter (MSV-LTR) which expresses a v-*fos* gene [16]. The c-H-*ras* and MSV-LTR promoters contain 13 and 2 GCTC motifs, respectively, within 350 bp of the transcription initiation site [31,44]. In this study we set out to understand the possible mechanism behind these observations considering the function of the *rep* protein as a DNA binding transcription factor. Here we present direct evidence of the interaction of a large AAV *rep* protein, expressed as a recombinant fusion protein, with the c-H-*ras* promoter at a region containing multiple GCTC motifs and its non-interaction with MSV-LTR which has only one GCTC motif. Because of its wide range of inhibitory effects on oncogenes, understanding the molecular mechanisms of these phenomena would eventually contribute to the anti-cancer therapy.

2. Materials and methods

2.1. Preparation of end-labeled oligonucleotide probes

The DNA oligomers used in the mobility shift assay were custom synthesized and ^{32}P-5′ end-labeled with T4 polynucleotide kinase using. To generate the AAV terminal repeats (TR) three separate oligomers were synthesized, annealed and ligated together prior to the kinasing. Together, these three oligomers form the full TR cruciform. Complementory strands of the sequences of c-H-*ras* promoter and of MSV-LTR were also annealed prior to the kinasing. The custom synthetic DNAs (purchased from Operon) for the AAV TR substrate included: AAV TR1, 5′TGAGGCCG-CCCGGGCAAAGCCCGGGCGTCGGGCGA-CCTTTGGTCGCCCGGCCTCAGTGAGC3′; AAV TR2, 5′CACTCGCTCGCTCGCGCGTC-TCTCCCTCACCGGTTGAGGTAGTGATCC-CCAAGGA3′; and AAV TR3, 5 ′GAGCGA-GCGCGCAGAGAGGGAGTGGCCAACTCC-CATCACTAGGGGTTCCT3′. The custom synthetic DNAs for the c-H-*ras* substrate included: c-H-*ras* A, 5′CTAGAACCCCGAGCTCGGCTC-CGGTCT3′; and c-H-*ras* B, 5′CTAGAGACC-

GGAGCCGAGCTCGGGGTT3′. The custom synthetic DNAs for the MSV-LTR substrate included:MSV-LTR A, 5′GCTGCCCAGCTCTATAAAAA3′; and MSV-LTR B, 5′TTTTTATGAGCTGGGCAGC3′.

2.2. Protein expression

The AAV *rep* open reading frame was cloned and expressed in *E. Coli* as a fusion protein with maltose binding protein as described previously [9]. The fusion protein, MBP-*rep*68Δ, was purified according to the protocol given by New England biololabs and the homogeneity was confirmed by SDS–polyacrylamide gel electrophoresis (data not shown).

2.3. Electrophoretic mobility shift assay (EMSA)

Assays were performed as previously described with minor modifications [20]. Briefly, assays were carried out with 1 ng of various 5′ end-labeled oligonucleotides which were incubated for 15 min at room temperature with 100–500 ng of MBP-*rep*68Δ in a final volume of 20 μl binding buffer (25 mM HEPES–KOH, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 2% glycerol, 0.25μg bovine serum albumin, 50 mM NaCl, 0.01% Nonidet P-40, 0.5μg of poly(dI-dC)). Samples were electrophoresed on 4.5% polyacrylamide gels (40:1 acrylamide-bis acrylamide weight ratio) with 5% glycerol in 0.5 × TBE buffer at 100 V for about 2.5 h. Gels were dried and autoradigraphed at −70°C. To promote the secondary structure formation of the individual oligonucleotides, end-labeled single stranded oligonucleotides were boiled for 2 min and chilled immediately. Experiments involving competition between radiolabeled substrate and unlabeled homologous substrate were performed as described in the figure legends.

3. Results

3.1. MBP-rep68Δ interacts with sequences of c-H-ras promoter

Earlier observations from our laboratory indicated that the large AAV *rep* gene product inhibited the transforming ability of c-H-*ras* but not MSV-FBR (v-*fos*) in focus formation assays. In an attempt to further investigate this phenomenon, EMSA experiments were devised to explore the possibility of *rep* protein, as an MBP-*rep* chimeric, directly interacting with c-H-*ras* sequences and also to confirm the proposed GCTC motifs are, in fact, the *rep* binding motifs. We identified 13 GCTC motifs within (nt −350 to +1 relative to the transcription initiation site) the *rep* sensitive c-H-*ras* promoter [31] (Fig. 1B) and two motifs in the *rep* resistant MSV-LTR promoter [44]. From the standpoint of the GCTC motifs, the most interesting region within the c-H-*ras* promoter included three such motifs (motifs 7–9, Fig. 1B) within an 11-base sequence (nt −330 to −320). The MSV-LTR promoter contained no comparable GCTC-rich clusters. However, one motif (motif 1, Fig. 1C) was provocatively located immediately adjacent to the TATA box of the LTR's promoter. Custom synthetic double stranded oligonucleotide substrates of these selected c-H-*ras* and MSV-LTR sequences were purchased and analysed for their ability to be recognized and bound by *rep*. Synthetic AAV TRs, which are known to bind *rep*, were also assayed (positive control) (Fig. 1A). As shown in Fig. 2, the band shift patterns indicate that *rep* does bind to the selected sequences from the c-H-*ras* promoter, although to a lesser extent than to the AAV TR. The Fig. 2 exposure does not show *rep* binding to the selected sequences from the MSV-LTR, however; on much longer exposures there is a very low level of band shift observable. Thus, the differential ability of MBP-*rep*68Δ to recognize and bind to the selected c-H-*ras* and MSV-LTR sequences is consistent with the differential sensitivity of the two promoters to inhibition by AAV *rep*. Non-chimeric MBP protein, missing *rep*, had no observable ability to band shift DNA (data not shown). Furthermore, it is observed in Fig. 2 that there are multiple high shifted bands, usually three, in the MBP-*rep*68-plus-TR-oligomer lane. This set of bands has been observed by others using either fully wild type *rep* or chimeric MBP-*rep*68Δ [9,20]. In Fig. 2, it is observed that the MBP-*rep*68Δ-plus-*ras*-oligomer lane also shows a very similar band shift pattern. This similarity is interesting considering that the AAV TR and the *ras* substrates are very different in size. Possibly, the size of the *rep* protein is the most significant determinant of the band positioning.

A

...A$_{-140}$GGAACCCCTAGTGATGGAGTTGGCCACTCCCTCTCTGCGCGCTCGCTCGCTCACTGAGGC$_{-86}$

B

TGTTCCCGGGAGAGC^{12}CC$_{-450}$GGGGCCCTGCTC^{11}GGAGATGCCGCCCCGGGCCCCCAGACAC$_{-300}$CGGCTC^{11}C

CTGGCCTTCCTCGAGC^{10}AACCCCGAGC^{9}TC^{8}GGCTC^{7}$_{-270}$CGGTCTCCAGCCAAGCCCAACCCCGAGAGGCCGCGGCCCT$_{-200}$ACT

GGCTC^{6}CGCCTCCCGCGTTGCTC^{5}CCGGAAGCCCCGCCC$_{-240}$GACCGCGGCTC^{4}CTGACAGACGGGCCGCTC^{3}AGCCAACCGGG$_{-200}$

GTGGGGCGGGGCCCGATGGCGCGCAGCCAATGGTAGGCCG$_{-160}$CGCCTGGCAGACGGACGGGCGCGGGGCGGGGCGTGCG

CAG$_{-120}$GCCCGCCCGAGTCTCCGCCGCCGTGCCCTGCGCCCGCAAC$_{-80}$CCGAGC^{2}CGCACCCGCCGCGGACGGAGC^{1}CCATG

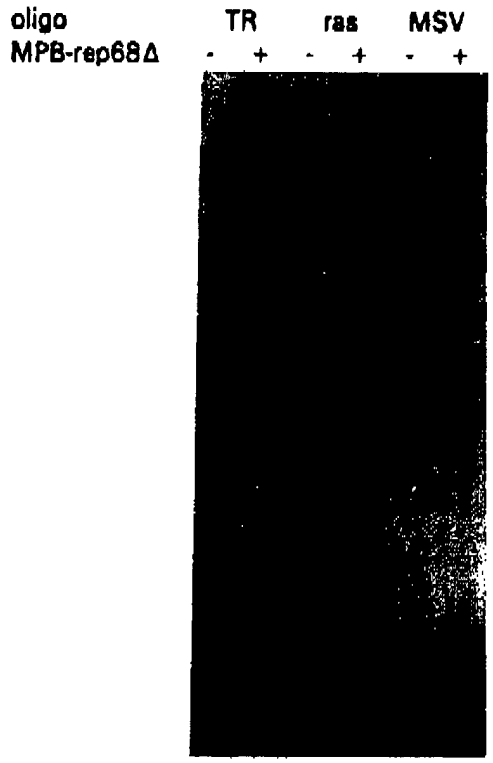
CGCGGGGC$_{-40}$GAACCGCGCGCCCCCGCCCCCGCCCCGCCCCGGCCTCGG^{+}C$_{+1}$CCCGGCCCTGG^{+}CCCCG^{+}GGGGCAG^{+}

C

AACCA$_{-80}$ATCA

GCTC^{3}GCTTCTCGCTTCTGTACCCGCGCTTATTGCTG$_{-40}$CCCAGCTC^{1}ATAAAAAGGGTAAGAACCCCACACTCGGC^{+}G$_{+1}$

Fig. 1. Features of The AAV terminal repeat (AAV TR) (A), c-H-*ras* promoter (B), and sequences of MSV-LTR promoter (C). Only those regions of the AAV TR which include GCTC motifs are included in the Figure. The sequences used to generate the substrates used in the EMSA assays are underlined. The transcription initiation sites are denoted by a supscript right hand arrow. The nucleotide positions, relative to the transcription initiation sites, are denoted by subscript numbers. The GCTC motifs, in bold and italics, are individualized by marked superscript numbers. GCTC motifs were highlighted and the selected sequences were underlined. The complete hairpin configuration of AAV TR was not depicted in the picture.



3.2. Non-labeled c-H-ras oligomer substrates, but not MSV-LTR, are able to effectively compete with c-H-ras/MBP-rep68Δ complexes

To demonstrate specific binding of MBP-*rep*68Δ to sequences within the c-H-*ras* promoter and also to further confirm its very weak interaction with the MSV-LTR sequences, we used unlabeled oligonucleotides as competitors in the EMSA assay. As shown in Fig. 3, cold c-H-*ras* oligonucleotide could effectively compete away its ^{32}P-labeled counterpart at the ratio of 10:1. This is evidenced by the disappearance of the high bands in the 10 ng *ras* competitor lane when compared with the adjacent lanes. In sharp contrast, cold MSV-LTR sequences did not compete with MBP-*rep*68Δ-plus-c-H-*ras* complex formation (Fig. 4).

Fig. 2. Electrophoretic mobility shift assay (EMSA) showing the interaction of MBP-*rep*68 with the AAV TR (TR), c-H-*ras* and MSV-LTR substrates. Probes indicated above the panel were incubated with 100 ng of affinity purified MBP-*rep*68 in the binding buffer as mentioned in Materials and methods. *Symbols:* (−) indicates oligonucleotide alone, (+) indicates oligonucleotide together with MBP-*rep*68Δ.

Fig. 3. EMSA assay demonstrating that unlabeled c-H-*ras* substrate effectively competes with ^{32}P end-labeled product of the same. Where indicated (+), 100 ng of MBP-*rep*68Δ is added. One nanogram of end-labeled c-H-*ras* oligonucleotide used. All other assay conditions were as described in Fig. 2.

Fig. 4. EMSA assay demonstrating non-effective competition of unlabeled MSV-LTR substrate with ^{32}P end-labeled c-H-*ras* substrate. Approximately 100 ng of MBP-*rep*68Δ was incubated with 1 ng of ^{32}P end-labeled c-H-*ras* substrate wherever indicated (+). Addition of increasing amounts cold competing MSV-LTR substrate is indicated above the panel.

These data (Figs. 3 and 4) are entirely consistent with the relative strength of the band shifting, reflecting MBP-*rep*68Δ's oligonucleotide affinity, observed in Fig. 2, with MBP-*rep*68Δ having a higher affinity for c-H-*ras* promoter sequences than the MSV-LTR promoter sequences.

3.3. Non-labeled c-H-ras oligomer substrates are unable to compete with AAV TR/MBP-rep68Δ complexes

We next verified that the affinity of MBP-*rep*68Δ was higher for the AAV TR than for the selected c-H-*ras* promoter sequences. In Fig. 5, we observed that the non-labeled c-H-*ras* oligomer could only very poorly interfere with ^{32}P-labeled AAV TR/MBP-*rep*68Δ complexes at levels in which cold c-H-*ras* oligos were able to strongly compete with *ras*/MPB-*rep*68Δ complexes. Thus, taken together, these data (Figs. 2–5) indicate that *rep* protein binds to the AAV TRs with the highest affinity, recognize the selected c-H-*ras* sequences with intermediate affinity, and only very weakly bind the selected MSV-LTR promoter sequences.

3.4. MBP-rep68Δ also binds to hairpined substrates with GCTC motifs

The AAV TR is a complex palindrome whose overall structure is described as a cruciform. It has been shown that this secondary structure is involved in *rep* protein recognition of the AAV TR

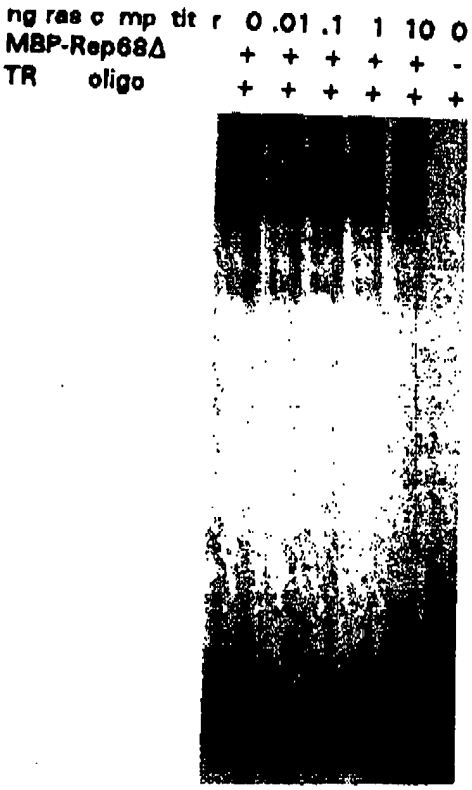
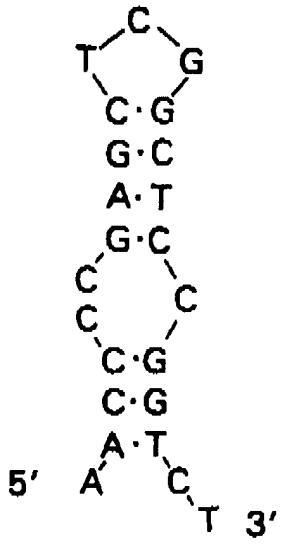


Fig. 5. EMSA assay demonstrating non-effective competition of unlabeled c-H-*ras* (ras) substrate with ^{32}P end-labeled AAV TR (TR). One hundred nanograms of MB-*rep*68Δ and 1 ng of end-labeled TR used in the binding assay with indicated amounts of cold c-H-*ras* oligo (+).



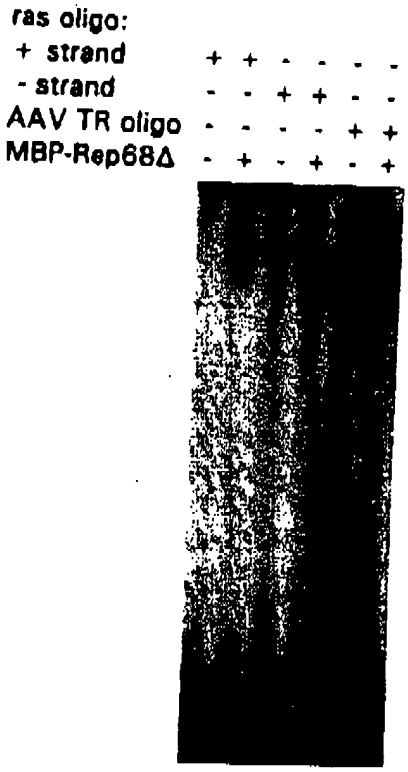


Fig. 7. EMSA assay demonstrating binding of 'snap back' secondary structure DNA of the c-H-*ras* substrates to MBP-*rep*68Δ. One nanogram of ^{32}P end-labeled single stranded oligonucleotide was boiled and chilled and incubated with 200 ng of the MBP-*rep*68Δ in the incubation mixture. Details of the lanes are depicted in the upper panel.

[20–22]. It would seem possible that the secondary structures of other DNAs may also affect *rep* binding. We further noticed that the c-H-*ras* substrate, due to the GCTC motifs (motifs 7 and 9, Fig. 1B) being present in opposite orientations, could form a hairpin loop (Fig. 6). To observe potential *rep* binding we boiled and then quick-chilled the positive and negative c-H-*ras* oligonucleotide strands separately to promote the formation of secondary structure. These hairpined substrates were assayed for *rep* binding activity in EMSA assay. As shown in Fig. 7, both the c-H-*ras*-positive and -negative

Fig. 6. Possible secondary structure within the c-H-*ras*-positive strand substrate. Base pairing between specific bases are denoted by dots. Upon boiling and quick chilling, the oligonucleotide is expected to snap back to this thermodynamically more favoured structure.

strand substrates bound AAV *rep*. Both of these hairpinned substrates and the MSV-LTR oligo contain only one duplex GCTC motif, yet only the hairpin containing substrates could significantly bind *rep*. These data suggest that sequences surrounding the GCTC motif play a role in substrate recognition.

4. Discussion

Although the inhibitory effects of large AAV *rep* on the transforming abilities of various oncogenes has been shown, the molecular mechanisms behind this phenomenon are not well understood. In this report, for the first time, we document the direct interaction of *rep*, as a fusion protein with MBP, with promoter sequences of a cellular gene, c-H-*ras*, which AAV *rep* is known to inhibit in co-transfection experiments [16,23,24]. These data imply a direct mechanism of action for *rep* protein inhibition of the c-H-*ras* promoter. Although these data give potentially significant insight into *rep*'s general mechanism of action, the details of the interaction remain unclear. The specific details of the mechanism of action could include: (i) direct interaction of *rep* with RNA polymerase II or its accessory proteins, (ii) steric hindrance of the binding of required transcription factors, or (iii) the chaperoning of negative regulatory proteins by *rep* to the c-H-*ras* promoter. These hypotheses are being further pursued in our laboratory.

Our data further support the hypothesis that GCTC motifs are the 'core' recognition sequence for *rep* binding. It has previously been shown that *rep* binds the AAV TR and the region of preferred AAV integration on human chromosome 19, both of which both contain three concatemeric GCTC motifs [9,20,45]. In this study we observed that MBP-*rep*68Δ differentially binds to three DNA substrates with affinities which are consistent with the number of GCTC motifs which are contained within. MBP-*rep*68Δ bound most strongly with the AAV TR (3 motifs) intermediately bound the selected c-H-*ras* sequence (2 effective, non-overlapping motifs), and only very weakly bound the selected MSV-LTR sequence (1 motif). These results are fully consistent with our earlier observations that the large AAV *rep* gene product selectively inhibits the c-H-*ras* promoter but not the MSV-LTR promoter in focus formation and transient CAT assays [16]. However, formal studies designed to specifically identify the most favored recognition site of *rep* need to be undertaken. Earlier reports differ on the importance of the cruciform/hairpinned configuration of the AAV TR in *rep* recognition [9,20–22]. The sequences surrounding the GCTC motifs must play a role in substrate recognition by *rep* as it bound hairpinned *ras* substrates with only one duplex GCTC motif (Figs. 6 and 7), but bound to a much less extent to the single motif MSV-LTR (Fig. 2). Thus, it appears that the multiple GCTC boxes are important for *rep* binding; however, secondary structure may promote the efficiency of binding.

In this study we observe a pattern of multiple shifted bands, usually three high bands, of *rep* with its natural TR substrate (Fig. 1). This is consistent with previous reports [20]. Our data in Fig. 2 suggest that the MBP-*rep*68Δ/TR complexes are similar to the MBP-*rep*68Δ/*ras* substrate complexes, as the same 3-band pattern, at similar positions, is evident. What this specifically means is unclear. Possibly, different stoichiometric associations, numbers of MBP-*rep*68Δ proteins, are binding the substrates, resulting in these multiple bands. Thus, more than one kind of protein–DNA complex appears to result between AAV MBP-*rep*68Δ and the TR or c-H-*ras* oligomers.

The finding that AAV *rep* protein directly binds to and affects at least one cellular promoter raises the possibility, or probability that other promoters are directly bound and affected by *rep*. We have recently published that AAV *rep* also negatively regulates both c-*fos* and c-*myc* [13]. We now have preliminary data that shows *rep* binds to selected sequences within the c-*fos* and c-*myc* promoters (R.B. Batchu and P.L. Hermonat, unpublished data). It seems likely that the pursuit of these interactions and mechanisms will eventually lead to the full understanding of AAV's general anti-proliferation affects on cells and on other viruses. This laboratory will continue to pursue this goal by addressing both the protein/DNA and protein/protein interactions of AAV *rep*.

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References

[1] Antoni, B.A., Rabson, A.B., Miller, I.L., Trempe, J.P., Chejanovsky, N. and Carter (1991) Adeno-associated virus Rep protein inhibits human immunodeficiency virus production in human cells. J. Virol., 64, 396–404

[2] Bantel-Schaal, U. and Zur Hausen, H. (1988) Adeno associated virus inhibit SV40 DNA amplification and replication of herpes simplex virus in SV40 transformed hamster cells. Virology, 164, 64–74.

[3] Beaton, A., Palumb, P. and Berns K.I. (1989) Expression from the adeno-associated virus P5 and P19 promotors is negatively regulated in trans by the Rep protein. J. Virol. 63, 4450–4454.

[4] Berns, K.I., Kotin, R.M. and Labow M.A. (1988) Regulation of adeno-associated virus DNA replication. Biochem. Biophys. Acta. 951, 425–429.

[5] Blacklow, N.R., Cukor, G., Kubrick, S. and Quinnan, G. (1978) Interactions of adeno-associated virus with cells transformed by herpes simplex virus. In: Replication of Mammalian Parvoviruses, pp. 87–97. Editors: D.C. Ward and P. Tattersal.

[6] Bohenzky, R.A., LeFebvre, R.B. and Berns, K.I. (1988) Sequence and symmetry requirements within the palindromic sequences of the adeno-associated virus terminal repeat. Virology, 166, 316–327

[7] Buller, R.M., Janik, J.E., Subring E.D. and Rose, J.A. (1981) Herpes simplex virus type 1 and 2 completely help adeno associated virus replication. J. Virol., 40, 241–247.

[8] Casto, B.C., Atchinson, R.W. and Hammon, W.McD. (1967) Studies on the relationship between adeno-associated virus type 1 and adenoviruses. I. Replication of AAV-1 in ceratin cell cultures and its effect on helper adenovirus. Virology, 18, 52–60.

[9] Chiorini, J.A., Weitzxan, M.D., Owens, R.A., Urcelay, E., Safer, B. and Kotin, R.M. (1994) Biologically active Rep proteins of adeno-associated virus Type 2 produced as fusion proteins in *E. Coli*. J. Virol., 68, 794–804.

[10] Cukor, G, Blacklow, N.R., Kibrick, S. and Swan, I.C. (1975) Effect of adeno-associated virus on cancer expression by herpes-transformed hamster cells. J. Natl. Cancer Inst., 55, 957–959.

[11] de la Maza, L.M. and Carter, B.J. (1981) Inhibition of adenovirus oncogenicity in hamsters by adeno associated virus DNA. J. Natl. Cancer. Inst., 67, 1323–1326.

[12] Georg-Fries, B., Biederlack, S., Wolf, J. and zur Hausen H. (1984) Analysis of proteins, helper dependence, and seroepidemiology of a human parvovirus. Virology. 134, 64–71.

[13] Hermonat, P.L. (1994) Down-regulation of the human c-*fos* and c-*myc* proto-oncogene promoters by adeno-associated virus rep 78. Cancer Lett., 81, 129–136.

[14] Hermonat, P.L. (1994) Adeno-associated virus inhibits human papillomavirus type 16: a viral interaction implicated in cervical cancer. Cancer Res., 54, 2278–2281.

[15] Hermonat, P.L. (1992) Inhibition of bovine papillomavirus plasmid DNA replication by adeno-associated virus. Virology, 189, 329–333.

[16] Hermonat, P.L. (1991) Inhibition of H-ras expression by the adeno-associated virus Rep78 transformation suppressor gene product. Cancer Res., 51, 3373–3377.

[17] Hermonat, P.L. (1989) The adeno associated virus rep78 gene inhibit cellular transformation induced by bovine papilloma virus. Virology, 172, 253–261.

[18] Hermonat, P.L. and Muzyczka, N. (1984) Use of adeno-associated virus as a mammalian DNA cloning vector: transduction of neomycin resistance into mammalian tissue culture cells. Proc. Natl. Acad. Sci., USA. 81, 6466–6470.

[19] Hermonat, P.L., Labow, M.A., Wright, R., Berns, K.I. and Muzyczka, N. (1984) Genetics of adeno-associated virus: isolation and preliminary characterization of adeno-associated virus type 2 mutants. J. Virol., 51, 329–339.

[20] Im.D.S. and Muzyczka, N. (1989) Factors that bind to adeno associated virus terminal repeats. J. Virol., 63, 3095–3104.

[21] Im.D.S. and Muzyczka, N. (1989) The AAV origin binding protein Rep 68 is an ATP dependent site specific endonuclease with DNA helicase activity Cell, 61, 447–457.

[22] Im.D.S. and Muzyczka, N. (1992) Partial purification of adeno-associated protein Rep78, Rep68, Rep52 and Rep40 and their biochemical characterization. J. Virol. 66, 1119–1128.

[23] Katz, E. and Carter, B.J. (1986) Effect of Adeno associated virus on transformation of NIH 3T3 cells by *ras* gene and on tumorigenicity of an NIH 3T3 transformed cell line. Cancer Res., 46, 3023–3026.

[24] Khleif, S.N., Myers, T., Carter, B.J. and Trempe, J.P. (1991) Inhibition of cellular transformation by the adeno associated rep gene. Virology, 181, 738–741.

[25] Klein-Bauernschmitt, P., zur Hausen, H. and Schlehofer, J.R. (1992) Induction of differentiation-associated changes in stablished human cells. J. Virol. 66, 4191–4200.

[26] Kotin, R.M., Linden, R.M. and Berns, K.I. (1992) Characterization of a prefered site on human chromosome 19 q for integration of adeno-associated DNA by non-homologous recombination. EMBO J., 11, 5071–5078.

[27] Kotin, R.M., Siniscalco, M., Samulski, R.J., Zhu, X., Hunter, L., Laughlin, C.A., McLaughlin, S., Muzyczka, N., Rocchi, M. and Berns, K.I. (1990) Site-specific integration by adeno-associated virus rep gene. Proc. Natl. Acad. Sci. USA, 87, 2211–2215.

[28] Labow, M.A., K.I. (1987) Adeno associated virus rep gene inhibit replication of an adeno-associated virus simian virus 40 hybrid genome in cos-7 cells. J. Virol., 62, 1705–1712.

]] Labow, M.A. and Graf, L.H. and Berns, K.I. (1987) Adeno associated virus gene expression inhibits cellular transformation by heterologous genes. Mol. Cell Biol., 7, 1320–1325.

]] Labow, M.A., Hermonat, P.L. and Berns, K.I. (1986) Positive and negative autoregulation of the adeno-associated virus type 2 genome. J. Virol., 60, 251–258.

] Lee, W. and Keller, E.B. (1991) Regulatory elements mediating transcription of the human Ha-*ras* gene. J. Mol. Biol. 220, 599–611.

] LeFebvre, R.B., Riva, S. and Berns, K.I. (1984) Conformation takes precedence over sequence in adeno-associated virus DNA replication. Mol. Cell Biol., 4, 1416–1419.

| Mayor, H.D., Drake, S., Stahmann, J. and Mumford, D.M. (1976) Antibodies to adeno-assocaited satellite virus and herpes simplex in sera from cancer patients and normal adults. Am. J. Obstet. Gynecol., 126, 100–104.

| Mayor, H.D., Drake, S., Stahmann, J. and Mumford, D.M. (1973) Influence of adeno-associated satellite virus on adenovirus-induced tumors in hamsters. Nat. New Biol., 241, 44–46.

McCarty, D.M., Ni, T.-H. and Muzyczka, N. (1992). Analysis of mutations in adeno-associated virus Rep protein in vivo and in vitro. J. Virol., 66, 4050–4057.

McPherson, R.A., Rosenthal, I.J. and Rose, J.A. (1985). Human cytomegalovirus completely helps adeno-associated virus replication. Virology, 147, 217–222.

Ostrove, J.M., Duckworth, D.H. and Berns, K.I. (1981) Inhibition of adenovirus transformed cell oncogenicity by adeno-associated virus. Virology, 113, 521–533.

[38] Rittner, K., Heilbronn, R, Kleinschmidt, J.A. and Sczakiel, (1992) Adeno-associated virus type 2 mediated inhibition of human immunodeficiency virus type 1 (HIV-1) replication: involve.

[39] Samulski, R.J., Zhu, X., Brook, J.D., Housman, D.E., Epstein, N. and Hunter, L.A. (1991) Targeted integration of adeno associated virus (AAV) into human chromosome 19. EMBO. J., 10, 3941–3950.

[40] Snyder, R.O., Im, D.-S., Ni, T., Xiao, X., Samulski, R.J. and Muzyczka, N. (1993) Features of adeno associated virus origin involved in substrate recognition by the viral rep protein. J. Virol., 67, 6096–6104.

[41] Snyder, R.O., Im, D.S. and Muzyczka, N. (1990) Evidence for covalent attachment of adeno-associated virus (AAV) rep proteins to the ends of AAV genome. J. Virol., 64, 6204–6213.

[42] Tratschin, J.D., Tal, J. and Carter, B.J. (1986) Negative and positive regulation in trans of gene expression from adeno-associated virus vectors in mammalian cells by a viral gene product. Mol. Cell. Biol., 6, 2884–2894.

[43] Trempe, J.P. and Carter, B.J. (1988) Regulation of adeno-associated virus expression in 293 cells: control of RNA abundance and translation. J. Virol., 62, 68–74.

[44] Van Beveren, C., Enai, S., Curran, T. and Verma, I.M. (1984) FBR murine osteosarcoma virus. Virology, 135, 229–243.

[45] Weitzman, M.A., Kyostio, S.R.M., Kotin, R.M. and Owens, R.A. (1994) Adeno-associated virus (AAV) Rep proteins mediate complex formation between AAV DNA and its integration site in human DNA. Proc. Natl. Acad. Sci. USA, 91, 5808–5812.

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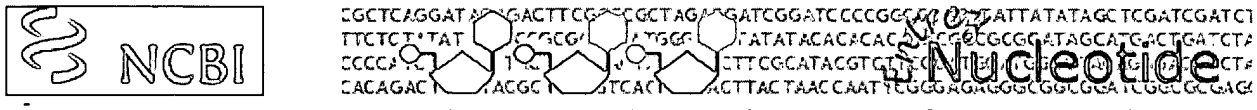


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VERSION     J01901.1  GI:209616
KEYWORDS    alternative splicing; complete genome; major coat protein.
SOURCE      Adeno-associated virus 2
  ORGANISM  Adeno-associated virus 2
            Viruses; ssDNA viruses; Parvoviridae; Parvovirinae; Dependovirus.
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  TITLE     Rescue of adeno-associated virus from recombinant plasmids: gene
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  JOURNAL   Cell 33 (1), 135-143 (1983)
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REFERENCE   2  (bases 1 to 4675)
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  JOURNAL   J. Virol. 45 (2), 555-564 (1983)
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HIV Sequence Database

TAT Alignments

HIV-1 TAT Nucleotide and Protein Alignments

- consensus nucleotide (4 kb) (text format)
  An alignment of DNA sequences, with one consensus sequence for each subtype of HIV-1. Only shows differences from first sequence
- consensus protein (2 kb) (text format)
  An alignment of protein sequences, with one consensus sequence for each subtype of HIV-1. Only shows differences from first sequence
- tat nucleotide (23 kb) (Intelligenetics format)
  An alignment of DNA sequences with a consensus for each subtype of HIV-1, plus several representative sequences for each subtype.
- tat nucleotide (31 kb) (text format)
  An alignment of DNA sequences with a consensus for each subtype of HIV-1, plus several representative sequences for each subtype. Only shows differences of representative sequences from consensus sequence
- tat protein (9 kb) (Intelligenetics format)
  An alignment of protein sequences with a consensus for each subtype of HIV-1, plus several representative sequences for each subtype.
- tat protein (2 kb) (text format)
  An alignment of protein sequences with a consensus for each subtype of HIV-1, plus several representative sequences for each subtype. Only shows differences of representative sequences from consensus sequence
- HIV-1 TAT sequences table (3 kb)
  A table providing the reference and GenBank accession number of each sequence used in the TAT alignments

HIV-2 TAT Nucleotide and Protein Alignments

- HIV-2 tat nucleotide (13 kb) (Intelligenetics format)
  An alignment of DNA sequences with a consensus for each subtype of HIV-2, plus several representative sequences for each subtype.
- HIV-2 tat nucleotide (17 kb) (text format)
  An alignment of DNA sequences with a consensus for each subtype of HIV-2, plus several representative sequences for each subtype. Only shows differences of representative sequences from consensus sequence
- HIV-2 tat protein (5 kb) (Intelligenetics format)
  An alignment of protein sequences with a consensus for each subtype of

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HIV-2, plus several representative sequences for each subtype.

- HIV-2 tat protein (6 kb) (text format)
  An alignment of protein sequences with a consensus for each subtype of HIV-2, plus several representative sequences for each subtype. Only shows differences of representative sequences from consensus sequence
- HIV-2 TAT sequences table (3 kb)
  A table providing the reference and GenBank accession number of each sequence used in the HIV-2 TAT alignments

SIV TAT Nucleotide and Protein Alignments

- SIVagm tat nucleotide (5 kb) (Intelligenetics format)
  An alignment of DNA sequences with a consensus for each species of SIVagm, plus several representative sequences for each species.
- SIVagm tat nucleotide (6 kb) (text format)
  An alignment of DNA sequences with a consensus for each species of SIVagm, plus several representative sequences for each species. Only shows differences of representative sequences from consensus sequence
- SIVagm tat protein (2 kb) (Intelligenetics format)
  An alignment of protein sequences with a consensus for each species of SIVagm, plus several representative sequences for each species.
- SIVagm tat protein (2 kb) (text format)
  An alignment of protein sequences with a consensus for each species of SIVagm, plus several representative sequences for each species. Only shows differences of representative sequences from consensus sequence
- SIVagm TAT sequences table (1 kb)
  A table providing the reference and GenBank accession number of each sequence used in the SIVagm TAT alignments

HMMER Global TAT Nucleotide and Protein Alignments

- HMMER tat nucleotide (12 kb) (text format)
  An alignment of DNA sequences generated using the HMMER program that includes a representative sequence of the subtypes of HIV-1, HIV-2 and SIV
- HMMER tat protein (7 kb) (text format)
  An alignment of protein sequences generated using the HMMER program that includes a representative sequence of the subtypes of HIV-1, HIV-2 and SIV

- HMMER tat nucleotide in MASE format (12 kb)

An alignment of DNA sequences generated using the HMMER program that includes a representative sequence of the subtypes of HIV-1, HIV-2 and SIV

- HMMER tat protein in MASE format (7 kb)

An alignment of protein sequences generated using the HMMER program that includes a representative sequence of the subtypes of HIV-1, HIV-2 and SIV

Questions or comments? Contact us at seq-info@t10.lanl.gov

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T--?--??----CCA?-?CC------AT- 63 CONSENSUS-U -----T--A-----C-----A------
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AT---------g-------Ca-A-cg---------- 140 CONSENSUS-F -A--C--T------
ACA-C?--G---------- 138 CONSENSUS-O -T--C--T--C----GA--C----AT-
G--A------?- 128 CONSENSUS-U -------T--------A--------AT-------C-G-G--------
CONSENSUS-CPZ -?--?--?--?---???-----T-A?--?--????-??-----CACA--A--------?
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CONSENSUS-B ----------.....----AG------a--g--------ga----c--ac-------gt-t--
----------.....----AG------A-CG------c-------G----------?- 191 CONSEN
AG------a--c--------g--g--C---C-------G?---- 203 CONSENSUS-F ----------.
G-----?C--ATA------G---T- 200 CONSENSUS-O ----------A.....?-??---?-?
G----- 181 CONSENSUS-U ----------.....----C-------A--T---------A---CG---
CPZ ----------???-A?C????-??A-??----?AC??--?--G?-?-----?-AA--A-----G--?-- 1
aTAcCAAAGCAAcCCtTaCCCcaaaCccaagGGgtC???cCGaCAGgCCcGaAAG
CONSENSUS-B c--t----------?Cct----gc---g-----A-...----------G----------
T----------g-----A-...----------G-g---------- 258 CONSENSUS-
G-----A----------G----A---------- 270 CONSENSUS-F G----------A--T
G?----------G--- 266 CONSENSUS-O G-----?-----T--C???--??C---???A--AAG..
G--?--?--- 236 CONSENSUS-U ----------T--C-------C-----GA-----...T----------
CONSENSUS-CPZ ?--?----?????--?--?--?-??--AG??--A?-...?A-????--??-?-GA---?
TGGagAGCAAGaCAgAGACAGATCgattCgcTTAG 301 CONSENSUS-B ----
CONSENSUS-C ----------?--?------c-----A---- 291 CONSENSUS-D ----------
CONSENSUS-F ----------G--??--?-----CG-G?-?----T?? 297 CONSENSUS-O --
G--?A?C--?GCC?C?AGG?TTCTTGC?AC?GTTGTACACGGA?CTC 294 CON
G----------A---- 306 CONSENSUS-CPZ ---?????-------?A?---?-????G???-

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M?PVDPnLEPWnHPGSqPtTaCskCYCK?CCwHCqlCFLnKGLGISYGrKKR..r
64 CONSENSUS-B -e----r----k------k---tn----k--f---v--tt----------..-Q--ra--dSq
CONSENSUS-D -d----------?-p-N--h--K--Y---v--it----------..-Q--rp--ggQ
CONSENSUS-F -EL-----D----------P-T-----R--F---W--TT----------..KQ-HR--
CONSENSUS-O -D----E?P--H----?-Q?P-NN----R--Y--YV--??------?-----...-???
CONSENSUS-U -D----K----------K---T-----K--Y--PV----------..-P--RS--
CONSENSUS-CPZ -D-?-????--?--???-?-?-NN-------Y--??--TK----------?-???---T
exon V exon CONSENSUS-A ipKQplPqtqg??ptgpkESkKkVeSKteTDrf?$ 95 C
Ls---?s-pr-D.----------rE----P?d? 99 CONSENSUS-D -----SS-pR-d-------?-----
CONSENSUS-F V----IS-AR-N.-----?---E----A??-P?--$ 96 CONSENSUS-O V-
S???-?RK.Q?RQE-QE??--K??GP?G?P????SC??CTR?S?Q$ 83 CONSENSUS-U
E----E----A-----D- 101 CONSENSUS-CPZ ??-??-?????-.?????K??-?-??--?????-?

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Protein reviews on the web

☐ **1:** Q92993 BLink, Domains, Links
Histone acetyltransferase HTATIP (60 kDa Tat interactive protein) (Tip60) (HIV-1 Tat interactive protein) (cPLA(2) interacting protein)
gi|30923328|sp|Q92993|TI60_HUMAN[4170731]

☐ **2:** Q13049 BLink, Domains, Links
Zinc-finger protein HT2A (72 kDa Tat-interacting protein) (Tripartite motif-containing protein 32)
gi|20178303|sp|Q13049|HT2A_HUMAN[4167729]

☐ **3:** Q15633 BLink, Domains, Links
TAR RNA-binding protein 2 (Trans-activation responsive RNA-binding protein)
gi|3334380|sp|Q15633|TRBP_HUMAN[4160145]

☐ **4:** P04613 BLink, Links
TAT protein (Transactivating regulatory protein)
gi|135347|sp|P04613|TAT_HV1MA[4150635]

☐ **5:** P04610 BLink, Links
TAT protein (Transactivating regulatory protein)
gi|135336|sp|P04610|TAT_HV1BR[4150634]

☐ **6:** P12506 BLink, Domains, Links
TAT protein (Transactivating regulatory protein)
gi|135334|sp|P12506|TAT_HV1Z2[4150633]

☐ **7:** P04606 BLink, Domains, Links
TAT protein (Transactivating regulatory protein)
gi|135329|sp|P04606|TAT_HV1B1[4150632]

☐ **8:** NP_758891 BLink, Domains, Links
tat protein [Simian immunodeficiency virus 2]
gi|27311172|ref|NP_758891.1|[4000395]

☐ **9:** NP_406123 BLink, Domains, Links
sec-independent protein translocase protein [Yersinia pestis]
gi|16122810|ref|NP_406123.1|[3941135]

☐ **10:** NP_056842 BLink, Domains, Links
tat protein [Human immunodeficiency virus 2]
gi|9628886|ref|NP_056842.1|[3738757]

☐ **11:** NP_042686 BLink, Domains, Links
alternative tat protein [Jembrana disease virus]
gi|9628099|ref|NP_042686.1|[3738081]

☐ **12:** NP_042687 BLink, Domains, Links
tat protein [Jembrana disease virus]
gi|9628096|ref|NP_042687.1|[3738078]

☐ **13:** NP_041252 BLink, Domains, Links
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gi|9627005|ref|NP_041252.1|[3737339]

☐ **14:** NP_040842 BLink, Domains, Links
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gi|9626551|ref|NP_040842.1|[3736993]

☐ **15:** AAP76567 Links
truncated tat protein [Human immunodeficiency virus 1]
gi|32261507|gb|AAP76567.1|[3599119]

☐ **16:** AAP76560 BLink, Links
tat protein [Human immunodeficiency virus 1]
gi|32261499|gb|AAP76560.1|[3599112]

☐ **17:** AAP76551 BLink, Links
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gi|32261489|gb|AAP76551.1|[3599103]

☐ **18:** AAP76542 BLink, Links
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gi|32261479|gb|AAP76542.1|[3599094]

☐ **19:** AAP76533 BLink, Links
tat protein [Human immunodeficiency virus 1]
gi|32261469|gb|AAP76533.1|[3599085]

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